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NITROGEN EXCHANGE BETWEEN PLANTS THROUGH COMMON MYCORRHIZAL NETWORKS

By

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Declaration

I declare that the substance of this thesis, to the best of my knowledge, has not already been submitted, in whole or in part, for a degree at this or any other university.

I certify that, to the best of my knowledge, any help received in preparing this thesis and all sources used have been acknowledged in this thesis.

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Abstract

Nitrogen (N) exchange mediated through common mycorrhizal networks (CMNs) was investigated in pure and mixed systems of *Casuarina cunninghamiana* and *Eucalyptus maculata*, and *Glycine max* (soybean) and *Sorghum bicolour* (sorghum). Both ^{15}N labeling and ^{15}N natural abundance ($\delta^{15}N$) studies were performed.

Seeds of all four species were aseptically germinated with or without mycorrhizal fungi or N_2-fixing bacteria on agar media in petri dishes. Seedlings were transplanted into three-compartment growth units and paired. 37 µm diameter nylon mesh, *RainSaver* crystals (high water holding capacity) and *N-serve* (nitrification inhibitor) prevented direct root contact, soil nutrient flow with water and nitrification, respectively.

At harvest, none of the controls was mycorrhizal or nodulated, while all originally non-mycorrhizal seedlings were colonised. Mycorrhizal hyphae penetration through the nylon mesh was directly observed and demonstrated with an Environmental Scanning Electron Microscope (ESEM). Colonisation of roots as high as 80% confirmed that common ectomycorrhizal or arbuscular mycorrhizal networks were established between pairs in all combinations.

Mycorrhization had significant effects on biomass production in both N_2-fixing plants (*Casuarina*, soybean) and non-N_2-fixing ones (*Eucalyptus*, *Sorghum*). Dry matter production was highest in both partners when N_2-fixing plants were mycorrhizal and nodulated. However, mycorrhization had little impact on N accumulation in eucalypts, but had a major effect in casuarinas, despite eucalypts

having nearly double the colonisation rate. Biomass was positively correlated with tissue N content in both species. The nodulated mycorrhizal casuarinas and their companion mycorrhizal eucalypts had the highest tissue N accumulation. Both biomass and total N in all N-receivers equalled those in N-donors, especially when nodulated casuarinas were N-receivers. The above trends were generally true for soybean and sorghum pairs. In addition, $\delta^{15}N$ values were negative in nodulated casuarinas, but positive in nodulated soybeans. Biological nitrogen fixation (BNF) contributed up to 50% and 40% of N in nodulated mycorrhizal casuarinas and soybeans, respectively.

From both ^{15}N labelling experiments and $\delta^{15}N$ analyses it was established that N-transfer occurred bidirectionally (two-way) between *Casuarina* and *Eucalyptus*, and between soybean and *Sorghum*. The percentages and amounts of N transferred, and the % of N in the receiver derived from the transfer (%NDFT) were generally significantly higher in the nodulated/mycorrhizal pairs than in the non-nodulated/mycorrhizal pairs. This occurred regardless of whether the nodulated N_2-fixing plants were 'N-donors' or 'N-receivers'. However, the %NDFT was always on the same scale regardless of the direction of N-transfer. The % and amount of N-transfer were also significant from non-N_2-fixing plants to nodulated N_2-fixing plants (with up to 50% biological N_2-fixation) rather than the reverse. Significantly higher bidirectional and net N-transfer were also found between the sole mycorrhizal and the nodulated/mycorrhizal pairs. These results indicated a net gain in N by N_2-fixing plants, but not by non-N_2-fixing ones.

The similar N transferred to non-N_2-fixing plants and to N_2-fixing ones in the sole mycorrhizal pairs indicated that two-way N-transfer could occur naturally between

any mycorrhizal plants, regardless of whether they were N_2-fixing plants or non-N_2-fixing ones, and that N resources could equally be reallocated between plants through mycorrhizae. The significantly greater intensity of bidirectional N-transfer in the nodulated mycorrhizal pairs showed that more substantial amounts of N could be shuttled between plants because of a generally greater physiological and ecological N demand in low-external-N-input conditions. These results therefore suggest that N_2-fixing capacity might not be a prerequisite for, but might affect the intensity of, this two-way N-transfer.

In addition to accessing N from soils directly by roots, the experiments suggest that N_2-fixing plants have two further strategies, N_2-fixation and mycorrhization, to satisfy their high N-demand, while mycorrhization alone can meet the needs for relatively low N-demand by non-N_2-fixing plants. Two 'mycocentric' N-transfer mechanisms are postulated to account for these differences. It seems that any plant that gives more N than it receives is an 'N-donor', while the opposite is true for an 'N-receiver'. If these mechanisms operate as these experiments have demonstrated and prove to be widespread, ideas about mycorrhiza-mediated N exchange and cycling in both agricultural and natural ecosystems may have to be re-evaluated, and concepts about nutrient cycling and energy exchange in plant communities may also have to be reformulated. Bidirectional N-transfer certainly has important implications for the nitrogen economy of N_2-fixation-based agricultural and natural ecosystems. In such ecosystems, the magnitude of mycorrhiza-mediated N-transfer and N movement seems to be determined by the dynamic four-way interactions between plant roots, mycorrhizal fungi, N_2-fixing bacteria, and N resource availability and requirements.

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AM	arbuscular mycorrhiza (e) (l)
BNF	biological nitrogen fixation
C	carbon
CMN(s)	common mycorrhizal network (s)
DW	dry weight
ECM	ectomycorrhiza (e) (l)
g	gram
GDH	glutamate dehydrogenase
GOGAT	glutamate synthetase
GS	glutamine synthetase
ha	hectare
HC(s)	hyphal compartment (s)
L	litre
N	nitrogen
N_2	atmospheric dinitrogen
na	not available
NDFT	nitrogen in the receiver derived from the transfer
NH_4^+	ammonium
NO_3^-	nitrate
P	phosphorus
t	ton(ne)
VAM	vesicular-arbuscular mycorrhiza (e) (l)
w/v	weight/volume

CHAPTER 1 LITERATURE REVIEW

1.1 Introduction

1.1.1 *General description of symbiosis*

The term "symbiosis" was first used by Frank in 1877 to describe the regular coexistence of different organisms such as fungi and algae in lichens. It was used as a neutral term that did not imply parasitism or mutualism. A decade later, De Bary (1887) used "symbiosis" to include parasite as well. But the meanings of both "symbiosis" and "parasite" were changed later on. Symbiosis was increasingly used for mutually beneficial associations between dissimilar organisms, and parasite and parasitism came to be almost synonymous with pathogen and pathogenesis (see Smith and Read, 1997). Symbiosis has been recently defined as "the obligatory cohabitation of two dissimilar organisms in intimate association, often, but not always, mutually beneficial" (The Soil Science Society of America, Inc., 2001).

This definition includes all associations ranging from *mutualistic*, in which all organisms involved are believed to derive benefit; to *parasitic*, in which one organism benefits to the disadvantage of another. A more precise definition of mutualism is that associations are *mutualistic* if the fitness (i.e. the ability to produce offspring) of the associated organisms is greater than that of their individual counterparts (Law and Lewis, 1983). In a symbiosis, the organism with the larger size is generally the host and the smaller is the symbiont. The symbiont can be external to the host (ectosymbiotic) or within it (endosymbiotic). The symbiosis is *obligate* for an organism that is unable to survive and reproduce without its living partner, and *facultative* if it is able to do so when its living partner is absent.

Symbiosis is wide-spread especially between higher plants and bacteria in N_2-fixing associations, between higher plants and fungi in most mycorrhizae, between algae and fungi in lichens, and between algae and coelenterates in corals. While it is not currently known whether symbiosis occurs between higher plants, the tripartite symbiosis among higher plant, N_2-fixing bacteria and fungi has been found in *Acacia*

(Reddell and Warren, 1987), *Albizia* (Binkley and Giardina, 1997), *Casuarina* (Reddell *et al.*, 1986; Vasanthakrishna and Bagyaraj, 1993) and *Leucaena* (Purcino *et al.*, 1986; Manjunath *et al.*, 1989). Symbioses are often found in nutrient limiting conditions and nutritional interactions play a key role in most of them. There are two important types of symbiosis: mycorrhizal and N_2-fixing symbiosis.

1.1.2 *Mycorrhizal symbiosis*

The word *MYCORRHIZA*, literally "fungus root", is derived from the Greek *MYKES* (fungus) and *RHIZA* (root). Mycorrhizae are highly evolved, mutualistic associations between soil fungi and plant roots. The partners in this association are members of the fungus kingdom (*Basidiomycetes*, *Ascomycetes* and *Zygomycetes*) and most vascular plants (Harley and Smith, 1983; Brundrett *et al.*, 1996; Smith and Read, 1997). Mycorrhizal plants have been found in every continent and in every major vegetation type. Depending on the plant and fungal species involved as well as distinct morphological characteristics, at least seven different types of mycorrhizal associations have been described: Vesicular-arbuscular mycorrhiza (VAM); Ectomycorrhiza (ECM); Ectendomycorrhiza; Orchid mycorrhiza; Ericoid mycorrhiza; Arbutoid mycorrhiza and Monotropoid mycorrhiza (Harley and Smith, 1983; Brundrett *et al.*, 1996; Smith and Read, 1997). However, Ectendomycorrhiza was not listed as a separate type by Martin *et al.* (2001), but treated as a "subtype" (Read, 2002). In addition, dual ECM and VAM associations have been found in *Alnus*, *Salix* and *Uapaca* in the world (Lodge and Wentworth, 1990; Zhao, 1995; Moyersoen and Fitter, 1999), and in *Acacia*, *Casuarina* and *Eucalyptus* in Australia (McGee, 1986; Reddell *et al.*, 1986; Reddell and Warren, 1987; Brundrett and Abbott, 1991; Brundrett *et al.*, 1996; Chen *et al.*, 2000).

VAM and ECM are the most common and the most economically important mycorrhizae so they are considered in more detail below. All fungi that form VAM belong to one fungal order (*Glomales*) in the Division *Zygomycota* (Morton and Benny, 1990). The origins and evolution of VAM fungi goes back 350-450 million years ago when plants were making the transition from water to land and gaining benefits from these associations to settle down (Simon *et al.*, 1993; Remy *et al.*,

1994). Of these VAM fungi, there are six genera: *Acaulospora, Entrophospora, Gigaspora, Glomus, Sclerocystis* and *Scutellospora,* containing approximately 150 species. The majority of these species are ubiquitous and non-host-specific. They can colonise the roots of most plants and there is hardly an ecosystem that does not contain VAM symbioses (Harley and Smith, 1983; Trappe, 1987; Brundrett *et al.*, 1996; Smith and Read, 1997; Kapulnik and Douds, 2000). However, they are all *obligate* biotrophs and have no saprobic ability at all in so far as we know. They are unable to survive under aseptic conditions and can only be reproduced in the presence of suitable living plant partners. Around 300,000 plant species form VAM from all plant divisions including Bryophytes, Pteridophytes, Gymnosperms and Angiosperms.

The main characteristic of VAM fungi is that hyphae can penetrate through the walls of root cortical cells to form vesicles, arbuscules, and external hyphal networks in the soil, and grow extensively within the cells of the cortex (Figure 1.1). This is the reason that this type of vesicular-arbuscular mycorrhiza was formerly called VAM. The term VAM, however, is no longer used. Instead, the term AM (arbuscular mycorrhiza) occurs in recent mycorrhizal literature, because many *Glomalean* species do not form vesicles.

AM development is highly structured and coordinated under the control of fungus and plant genes (Peterson and Bonfante, 1994). Roots colonisation can be initiated by infected root fragments, extraradical hyphae or spores. The fungus penetrates through the plant epidermis, sometimes through root hairs, and grows between and/or within cortical cells. However, it does not penetrate the endodermis and thus never enters into the vascular tissue. Normally, AM fungi occupy two matrices in the soil, the intra- and extra-radical matrices. The intraradical hyphae will often penetrate cortical cells to form haustorial structures called arbuscules. Being the plant/fungus interface and the sites of inorganic nutrient transfer from fungus to plant, arbuscules are modified hyphae that have branched many times to increase their surface area, which makes them effective structures for potential nutrient uptake and translocation. The extraradical matrix consists of a complex mycelial network and spores (Friese and Allen, 1991; Smith and Read, 1997).

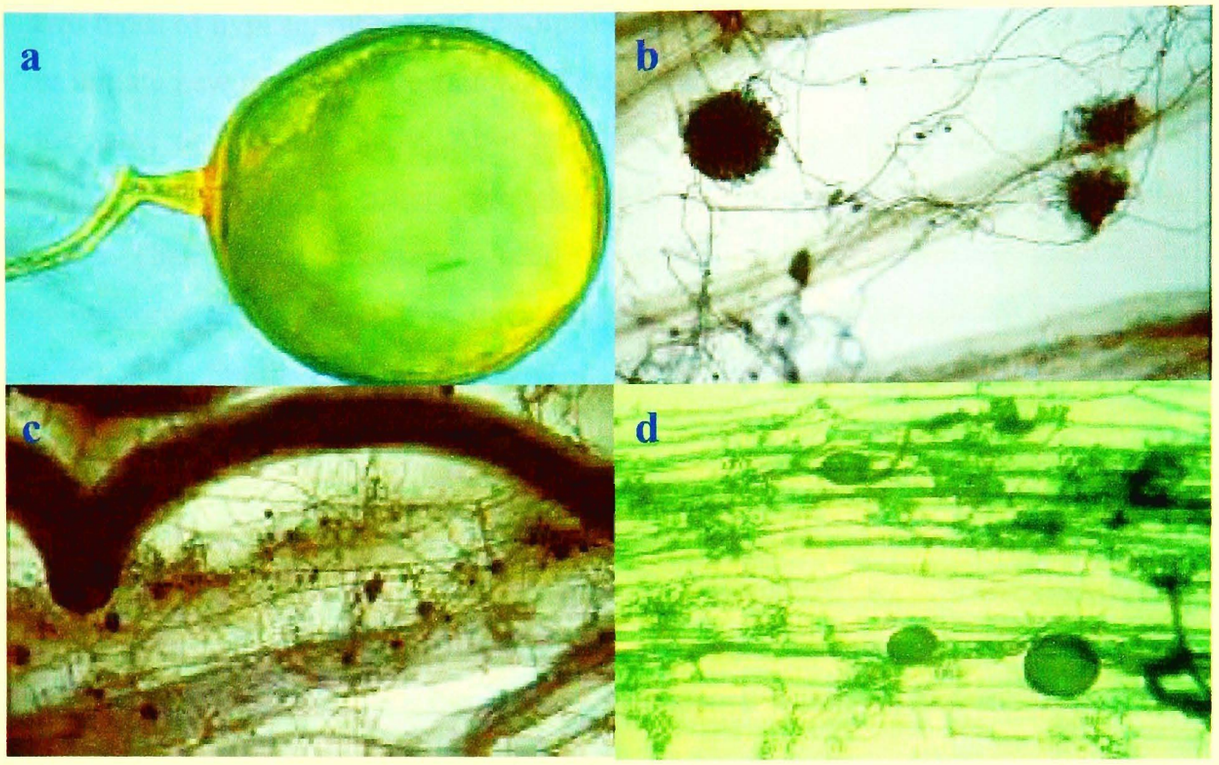

Figure 1.1. A single extramatrical spore of *Glomus mosseae* (a); AM in leek roots: monosporic sporocarps with hyphal peridium (b) and extramatrical mycelium (c); AM in ginger roots: hyphae, arbuscules and vesicles (d). Colonised by *G. mosseae* and stained with Chlorazol Black E. Adapted from http://mycorrhiza.ag.utk.edu.

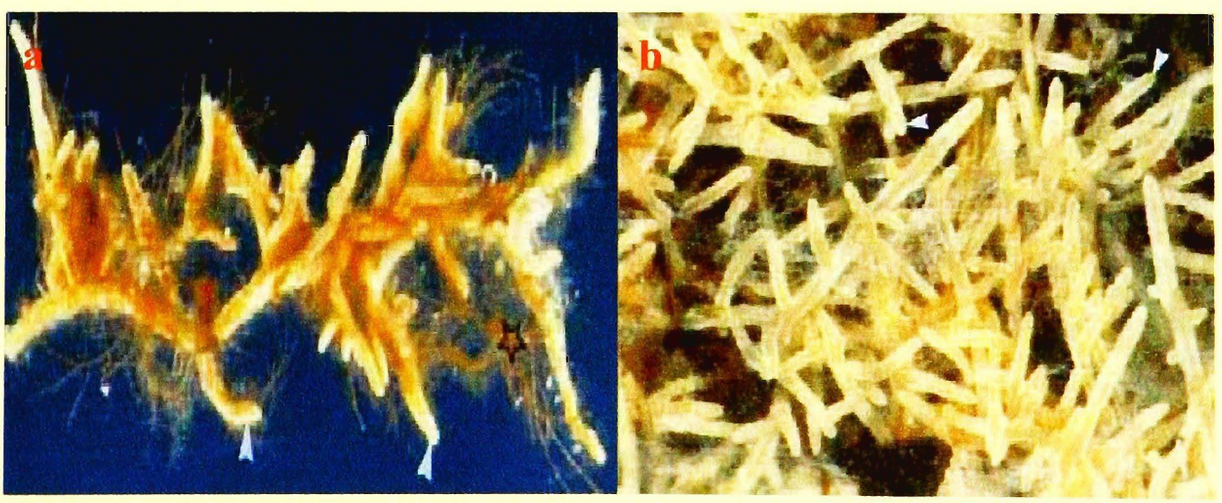

Figure 1.2. ECM associations of *Eucalyptus maculata* (spotted gum) with *Astraeus pteridis*: with relatively unbrached ECM (arrows) and attached mycelial strands (stars) (a), and *E. globulus* (Tasmanian blue gum) with *Lacearia* sp. (b). Adapted from http://mycorrhiza.ag.utk.edu.

The hyphae of AM fungi are typically aseptate and reproduce asexually. There are two types of hyphae: runner and absorptive. Runner hyphae grow along root systems to initiate new points of infection and function as a bridge between root segments of the same or different plants. Absorptive hyphae develop from runner hyphae, are highly branched and penetrate through soil pores where bare roots cannot normally reach. Their hyphal tips actively take up inorganic nutrients from soil, particularly phosphorus (P), which is then translocated to the plant partner (Smith and Smith, 1990, 2001; Smith *et al.*, 1994; Frey *et al.*, 2000; Kapulnik and Douds, 2000).

In contrast to AM fungi, ECM fungi are *facultative* biotrophs with >5,000 species in the division *Dikaryomycota,* but their partners are mostly limited to woody trees (Harley and Smith, 1983; Molina *et al.*, 1992; Brundrett *et al.*, 1996; Smith and Read, 1997). Most ECM fungi are *Basidiomycetes* and some *Ascomycetes*. Their hyphae are regularly septate and often produce sexual fruiting bodies so that they can be cultured and reproduced without living plant partners. Many ectomycorrhizal fungi are host specific for only a select group of plants, often within the same genus. For example, *Suillus lackei* associates only with *Pseudotsuga menziesii* (Douglas-fir), *S. grevillei* only with *Larix* (larch). However, other ECM fungi, such as *Cenococcum geophilum, Pisolithus tinctorius* or *Thelephora terrestris*, can colonise a broad range of temperate, subtropical and tropical trees.

The morphological characteristics of ECM are also dramatically different from those of AM (Peterson and Bonfante, 1994) (Figure 1.2). Ectomycorrhizal roots are thicker and have altered branching patterns. The lateral roots are characterised by a dense mycelial sheath around the roots which is called the mantle. The mantle often is differently coloured so that it can be easily distinguished from a non-mycorrhizal root. From the developing mantle, hyphae penetrate the root tissues, but not the cells, forming a mycelial network (fungal envelope) around each cortical cell. This unique, highly branched network is called the Hartig net – the plant/fungus interface, where solutes are translocated between the partners, carbohydrates from plant to fungus and inorganic nutrients from fungus to plant. ECMs involve an intimate association of the host plant's root tissue and the fungus, and fungal tissues extend into the soil as individual hyphae and as more complex strands or extraradical hyphal networks. The external hyphae can take up mineral nutrients from the soil and transport them into

the host root (Marschner and Dell, 1994, Smith *et al.*, 1994). Ectomycorrhizal associations can therefore be of great potential benefit to the host plant in nutrient limited systems (Allen, 1991, 1992; Read, 1991; Smith and Read, 1997).

Mycorrhizal fungi generally benefit their host plants by

1. increasing the physiologically absorbing surface area of the root system;
2. increasing the ability of plants to capture water and inorganic nutrients such as nitrogen (N), P, or other essential elements from the soil;
3. increasing the tolerance of plants to drought, high soil temperature, and extremes of soil acidity caused by high levels of metals such as sulfur, manganese, and aluminium;
4. providing protection from certain plant pathogenic fungi and nematodes that attack roots; and
5. modifying the transpiration rates and the composition of rhizosphere microflora by excretion of chelating compounds or ectoenzymes and other enzymes (Ho and Trappe, 1975; Smith and Read, 1997; Martin *et al.*, 2001).

In return for these benefits, the fungus receives carbohydrates (up to 20% of net photosynthate is allocated to AM or ECM fungi), vitamins and other nutrients supplied by the plant (Smith and Read, 1997). Meanwhile, awareness of mycorrhizal fungi (mostly in ECM and ericoid mycorrhizae) as users of organic N sources has been increasing in recent years (Nasholm *et al.*, 1998; Wallenda and Read, 1999; Lipson and Nasholm 2001; Nasholm and Persson, 2001). In addition, some rhizospheric bacteria, termed Mycorrhizal Helper Bacteria (MHB), do enhance the formation of mycorrhizae (Garbaye, 1994; Smith and Read, 1997), but there is no evidence that they play a direct role in nutrient transfer between plants.

1.1.3 *Nitrogen fixing symbiosis*

Nitrogen is the mineral nutrient most needed by plants and it often limits plant growth. Some plant species have formed mutualistic symbioses with N_2-fixing *prokaryotes*. Those organisms that can directly utilise the inert atmospheric dinitrogen (N_2) as an N source, are called "*diazotrophs*", and belong to the kingdoms

eubacteria and *archaebacteria*. They are able to live independently on soil N for growth. Inside the root nodules, however, the bacteria are supplied with carbon (C) from the host and are sheltered from competition with other organisms. The most important mutualistic symbioses are root nodule symbioses, mainly formed by members of the genera *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium* on legumes, and by the genus *Frankia* on some non-leguminous actinorhizal plants. The reduction of N_2 to ammonium (NH_4^+) which occurs in N_2-fixing organisms is catalysed by an enzyme complex called nitrogenase. A minimum 16 ATP and $8e^-$ are needed for a reduction of one molecule of N_2. The ratio of $^{15}N/^{14}N$ (0.003676) is very constant in the atmosphere, and soil often contains greater amounts of ^{15}N because of discrimination against ^{15}N during any physiological and biochemical process. Thus, a plant that contains fixed N from the air through N_2-fixing symbiosis will have less ^{15}N in its total N than if it obtains N only from soil sources. Thus quantitative isotope ratio measurements can assess the contribution of N_2-fixation to total N in the plant (Knowles and Blackburn, 1993). It is estimated that N_2-fixation could range from one to a few kg ha^{-1} $year^{-1}$ in lichens and free living bacteria (Sprent and Sprent, 1990), and up to a hundred or possibly a few hundred kg ha^{-1} $year^{-1}$ in legumes and actinorhizal plants (Dixon and Wheeler, 1983; Peoples and Herridge, 1990; Schwintzer and Tjepkema, 1990; Peoples and Herridge, 1999).

Symbiotic N_2 fixation in agriculture can be attributed mainly to legumes – the plants in the *Leguminosae* (Allen and Allen, 1981; Elkan, 1992; Spaink *et al.*, 1998; Sprent, 2001). It is estimated that *Leguminosae* contains more than 200 genera, and 20,000 species, which ranges from small plants such as the clover to the large trees such as *Acacia* species. Approximately 80% of them can fix N_2 from the atmosphere with *Rhizobiacea,* either with *Azorhizobium, Bradyrhizobium, Rhizobium, Mesorhizobium* or with *Sinorhizobium* in root nodules. The important agricultural legumes can be divided into three groups: crop legumes that are grown for their commodities; forage legumes that may be grazed or harvested for animal fodder; and trees or shrubs in agroforestry systems (Werner, 1995). Worldwide, about 1.5 million km^2 of land are cultivated with crop legumes, mainly *Glycine max* (soybean), *Phaseolus vulgaris*

(common bean), *Pisum sativum* (pea), *Arachis hypogaea* (groundnut) and *Cajanus cajan* (pigeon pea). The annual harvests of crop legumes are ~200 million tonnes, which provides the plant protein source for human and animal consumption, or vegetable oil and other raw materials. The areas covered by forage legumes, mainly *Trifolium* (clover), *Lotus* (trefoil), *Medicago* (lucerne), *Macroptilium* (bush bean) and *Mimosa* (mimosas) are even larger, ~30 million km^2 of grassland in the five continents. The third group consists of the genera *Acacia*, *Albizia*, *Alnus*, *Leucaena* and *Robinia*, which are mostly used as timber, fuelwood or craftwood, and in the pharmaceutical industry which extracts antibacterial and antifungal agents or food additives and other substances.

There are five genera in the *Rhizobiaceae*: *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* (Elkan, 1992; Spaink *et al.*, 1998; Sprent 2001). All of them are characterised by a gram-negative cell wall structure. Cells are generally rod-shaped, non spore-forming and motile with differently arranged flagella. All rhizobia are aerobic bacteria that persist saprophytically in the soil. Host-symbiont recognition initially occurs in the rhizosphere. The plant is usually infected through root hairs, which respond to bacterial signals by altering their growth pattern to trap potential symbionts. The enzymes from the bacteria degrade part of the cell wall and allow bacteria entry into the root-hair cell itself, which lead the root hair to produce a threadlike structure called the infection thread, that contains specific plant glycoproteins and other nodule-specific proteins. The bacteria multiply extensively inside the thread, which extends inwardly and penetrates through and between the cortex cells. In the inner cortex cells, the bacteria are released into the cytoplasm and stimulate some cells (especially tetraploid cells) to divide. Each enlarged, non-motile bacterium is referred to as a bacteroid. These result in a proliferation of tissues, eventually forming a unique root nodule.

In addition to infection via root hairs, the direct penetration and the crack entry also exist in some legume species. The direct penetration refers to the rhizobia that directly penetrate the primary cell wall at the junction of two epidermal cells, and no infection threads are observed in woody legumes that have no or very few root hairs

(de Faria *et al.*, 1988). Crack entry refers to entry by rhizobia into roots between two epidermal cells at the root surface (Boogerd and van Rossum, 1997). The key difference is the necessity for a 'wound' at the root surface before penetration of rhizobia can occur. This wounding is caused by the emergence of lateral roots, as nodules are found only at the junctions between lateral and main roots. In general, it is not easy to distinguish the mechanisms of these two infection pathways.

A typical root nodule cell contains several thousand bacteroids. The nodule also contains a protein called leghaemoglobin, which gives legume nodules a pink colour due to its prosthetic haem group. As an oxygen-binding plant protein, leghaemoglobin increases the flux and transport of O_2 moving through the plant cytoplasm into the bacteroids while controlling the concentration of free O_2, which is essential for bacteroid respiration. N_2-fixation in root nodules occurs directly within the bacteroids. The host plant provides bacteroids with carbohydrates, which they oxidise to generate the ATP and reductant, and from those they obtain energy. These carbohydrates are first formed in leaves during photosynthesis and then are translocated through the phloem to the nodules. Sucrose is the most abundant carbohydrate translocated, at least in legumes. Some of the electrons and ATP obtained during oxidation by the bacteroids are used to reduce N_2 to NH_4^+, which is catalysed by nitrogenase. The reduced NH_4^+ is first released from the bacteroids and is then assimilated into glutamine in the nodule cytosol. Depending on the form in which fixed N is exported from the nodule, legumes can be grouped into two classes: the producers of either amides (glutamine and asparagine) such as *Cicer arietinum* (chickpea), *Medicago sativa* (lucerne), pea and some *Acacieae* species, or ureides (allantoin and allantoic acid) such as *Vigna unguiculata* (cowpea), pigeon pea, soybean and some *Desmodieae* (desmodium) species. Both amides and ureides are transported from the nodule primarily through the xylem to the rest of the plant for further incorporation into other amino acids which occur in the plant cytoplasm and the plastids. There is little difference in energy demands between amides and ureides when the overall costs of synthesis and subsequent reassimilation are considered (Sprent and Sprent, 1990), though the solubility of ureides is less than that of amides.

In addition to the leguminous plants, about 200 angiosperm species from 25 genera, 8 families and 7 orders have been found to form nodule symbioses with N_2-fixing actinomycetes belonging to the genus *Frankia* and thus to be called non-leguminous (*Frankiaceae*) symbiosis (Benson and Silvester, 1993; Huss-Danell, 1997). With few exceptions, almost all of these species are perennial dicots, woody shrubs or trees, from eight different families (*Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae* and *Rosaceae*) (Tjepkema *et al.*, 1986; Schwintzer and Tjepkema, 1990; Pawlowski, 1999). They are named actinorhizal plants from *actino* in *actinomycete*, and from *rhiza* in the Greek word for root. Actinorhizal plants are found on every continent except Antarctica and in most climatic zones. They typically grow on disturbed marginal soils and are pioneer species early in successional plant community development, such as *Dryas* species in arctic tundra; *Casuarina*, *Hippophae*, *Myrica* and *Elaeagnus* species in coastal dunes; *Alnus* and *Myrica* species in riparian zones; *Alnus* and *Dryas* species in glacial till; *Casuarina*, *Ceanothus*, *Cercocarpus*, *Comptonia*, *Cowania* and *Purshia* species in chaparral and xeric zones; *Alnus* species in alpine zones; *Alnus*, *Casuarina*, *Coriaria* and *Shepherdia* species in forests (Benson and Silvester, 1993; Huss-Danell, 1997). Globally, especially wherever indigenous legumes are absent or rare, actinorhizal plants have potential applications in soil amelioration and reforestation, as fuelwood, timber and pulp, and as windbreaks (Diem and Dommergues, 1990; Dommergues, 1997; Huss-Danell, 1997) or even for addressing pyrodenitrification (Crutzen and Andreae, 1990).

The symbiont *Frankia* is a gram-positive, filamentous bacterium belonging to the family *Frankiaceae* within the order actinomycetes (Lechevalier and Lechevalier, 1990). Speciation in *Frankia* is not yet clear and within the genus different isolates are classified. Almost all taxa in the genus *Frankia* are characterised by hyphae, sporangia and vesicles. The hyphae are branched with a diameter of 0.5 to 1.5 μm and the mature vesicle is spherical with a diameter of 2 to 4 μm. Both the hyphae and the mature vesicles are septate (Benson and Silvester, 1993). When actinorhizal plants, such as *Alnus* and *Casuarina* seedlings are excavated from soil containing *Frankia*, numerous small, multilobed, coralloid-type, amber or whitish nodules are found on their root systems. The oldest and biggest nodules are close to the stem

base and the youngest are on the distal parts of the root system. A *Casuarina* nodule may reach a size of around 5 – 10 mm in diameter and a weight of about 1 g in dry matter. It has been reported that *Frankia* produce sporangia and vesicles as soon as the microorganism escapes from the mother nodule. This indicates that the formation of *Frankia* structures inside the nodule may be under host control (Diem and Dommergues, 1990). In general, the N_2-fixation rates of *Frankia*–actinorhizal plant symbioses are comparable to those of *Rhizobium*–legume symbioses (Schwintzer and Tjepkema, 1990; Huss-Danell, 1997). The form in which fixed N is transported is also host-dependent as seen in legumes (Schubert, 1986; Huss-Danell, 1990). For example, amides, predominantly asparagine, are transported in most actinorhizal plants, while citrulline (technically a ureide) is transported in some *Alnus* and *Casuarina* species.

1.1.4 *Benefits from N_2-fixing to non-N_2-fixing plants*

The N_2-fixing plant can fertilise directly the soil and indirectly neighbouring plants through above- and below-ground litter, through root exudates and leakage from leaves and roots. When N_2-fixing plants are grown in mixed plantations with non-N_2-fixing plants, an increase in both growth and yield of the non-N_2-fixing plants is often found (Hibbs and Cromack Jr., 1990, Fujita *et al.*, 1992; Chalk, 1998). Moreover, the legume/non-legume intercrops, on average, yield more efficiently per unit land with higher area- × -time equivalence ratios than intercrops of two legume or two non-legume species (Hiebsch and McCollum, 1987).

Compared to pure cultivation system, these possible benefits obtained by cereals may partly be due to transfer of the symbiotically fixed leguminous N, either through release from nodulated roots, decomposition of dead nodule and root tissue in the soil (Ledgard and Steele, 1992; Dubach and Russelle, 1994; Chalk, 1996a, b; 1998; Ledgard, 2001; Paynel *et al.*, 2001), or through mycorrhizal hyphal uptake and translocation (Miller and Allen, 1992; Marschner and Dell, 1994; Smith and Read, 1997; Kapulnik and Douds, 2000), or through common AM linkages from legumes to grasses (Bethlenfalvay, 1991; Frey and Schuepp, 1992, 1993; Johansen and Jensen, 1996). Similar observations have also been made through common ECM linkages from the actinorhizal N_2-fixing *Alnus* to non-N_2-fixing *Pinus* (Arnebrant *et*

al., 1993; Ekblad and Huss-Danell, 1995). Thus N_2-fixing plants (N-donors) and non-N_2-fixing plants (N-receivers) provide good systems for investigating nitrogen transfer (N-transfer). N-transfer can increase productivity of intercrops and forests without increasing the use of nitrogenous fertilisers if properly managed and quantified (Chalk, 1996a, b; 1998; Binkley and Giardina, 1997). However, the roles of mycorrhizal hyphae in direct N-transfer were somewhat inconclusive (Smith and Read, 1997), and it is not yet clear whether such transfer can be large enough to contribute significantly to the N status of the N-receiver in agricultural or natural ecosystems (Newman, 1988; Newman *et al.*, 1992). Other factors that may contribute to the positive effects of N_2-fixing plants on neighbouring plants in mixed plantations may involve lower competition for soil N (Danso *et al.*, 1993a) and effects on soil conditions generally (Bormann *et al.*, 1994). Therefore, studies are needed to identify the pathways of N-transfer and whether mycorrhizal hyphae play a direct role in facilitating this N-transfer in order to maximise such benefits.

1.2 Methods for Investigating Nitrogen Transfer between Plants

1.2.1 *Background*

Nitrogen has two stable isotopes, ^{14}N and ^{15}N, and four radioactive isotopes, ^{12}N, ^{13}N, ^{16}N and ^{17}N. The short half-life (0.1 second $\rightarrow$ 10 minutes) of radioactive isotopes of N makes them unsuitable for investigating most of the plant physiological processes. Of the two stable isotopes, ^{14}N is more abundant, accounting for ~99.6337% of atmospheric N whereas ^{15}N ~0.3663%. Since the ratio of $^{15}N/^{14}N$ (0.0036765) in the atmosphere is very constant, atmospheric N_2 is used as the standard for ^{15}N natural abundance ($\delta^{15}N$) analysis (Mariotti, 1983).

At present, the main plant physiological process investigated using the ^{15}N isotope is N_2 fixation. Both ^{15}N labeling or ^{15}N dilution methods (Peoples *et al.*, 1990; Knowles and Blackburn, 1993; Hardarson, 1994; Ladha and Peoples, 1995; Huss-Danell, 1997; Carranca *et al.*, 1999; Peoples and Herridge, 1999; Busse, 2000; Unkovich and Pate, 2001) as well as ^{15}N natural abundance methods (Shearer and Kohl, 1986; Virginia *et al.*, 1989; Handley and Raven, 1992; Unkovich *et al.*, 1994, 2001; Nilsen

and Orcutt, 1996; Handley and Scrimgeour, 1997; Högberg 1997; Boddey *et al.*, 2000; Tjepkema *et al.*, 2000) can be used for quantifying the relative contribution of biological nitrogen fixation (BNF) to whole-plant N accumulation. This application is possible because the N_2-fixing enzyme nitrogenase has minimal isotopic discrimination, and thus the fixed N mirrors closely the isotopic composition of atmospheric N (Knowles and Blackburn, 1993; Nilsen and Orcutt, 1996). ^{15}N enrichment has also been used to investigate N-transfer from N_2-fixing to non-N_2-fixing plants (Fujita *et al.*, 1992; Stern, 1993; Chalk, 1996a, b; 1998). ^{15}N natural abundance studies have also been tried for this purpose (Binkley *et al.*, 1985; van Kessel *et al.*, 1994; Kohls *et al.*, 1994).

The theoretical base for this is that ^{15}N will be discriminated against compared with ^{14}N during any physiological process because of its greater atomic mass. In the ^{15}N enrichment method, a source of N that is enriched in ^{15}N is used to follow events of N metabolism or N cycling in the ecosystem. This technique usually involves a large enrichment of ^{15}N over the background, making the measurement of isotope effects easy because the difference between the isotopic compositions of the source and the plant is large. In the ^{15}N natural abundance method, the natural abundance of ^{15}N of a sample is compared with that of the atmosphere or the soil. Studies of ^{15}N natural abundance involve very small ^{15}N concentrations and tiny differences between the sample and the atmosphere.

Estimates of total N transfer from N-donor to N-receiver are based on the assumption that proportions of labeled and non-labeled N are equally transferred. The percentage of total donor-N transferred to the receiver ($\%N_{transfer}$) is then estimated from the ratio between labeled N in the N-receiver and total labeled N in both the N-receiver and the N-donor (Ledgard *et al.*, 1985; Giller *et al.*, 1991; Ikram *et al.*, 1994; Johansen and Jensen, 1996). This aspect has been studied by supplying ^{15}N enriched fertiliser directly to the growth media and the donor root, with or without a split-root system; or to the donor plant, either by exposure to $^{15}N_2$, or by foliar spray or petiole injection of labeled ^{15}N (NH_4^+, NO_3^- or urea) solution (Chalk, 1996b; Chalk and Smith, 1997). For investigating N-transfer in mycorrhizal plants, a 20-50 μm nylon or stainless steel mesh is usually used to avoid direct contact of roots but permit

hyphal connection. The most satisfactory method for determining the fate of fixed N is to use ^{15}N-enriched N_2 (McNeill and Wood, 1990; Chalk, 1996b; Chalk and Smith, 1997), since ^{15}N detected in the donor is derived solely from N_2 fixation. But it requires enclosure of the leaf and/or root system within sealed growth chambers to get a ^{15}N-enriched atmosphere in which photosynthesis is difficult to control. On the other hand, because any addition of labeled fertiliser would perturb the N balance of the soil and invalidate the N_2-fixation measurement, use of the ^{15}N natural abundance method to investigate the role of mycorrhizae in N-transfer is increasing (Pate *et al.*, 1993; Smith and Read, 1997; Evans, 2001; Dawson *et al.*, 2002).

1.2.2 *Enriched ^{15}N labeling method*

The failure of the acetylene reduction technique arose in the mid-1960s to predict accurately the total N contribution from N_2 fixation led to a resurgence of interest in the now classical ^{15}N enrichment or dilution techniques which had been developed by McAuliffe *et al.* (1958) in the late-1950s (Hunt and Layzell, 1993). Assuming that the soil N cycling process is equivalent (mineralisation, denitrification, etc.) in the plant growth media, the original ^{15}N dilution technique and later modifications (Fried and Middleboe, 1977; Chalk, 1985; Ledgard *et al.*, 1985; Chalk and Smith, 1994), are based on the addition of ^{15}N-enriched fertiliser to the growth medium of N_2-fixing plants and on the detection of ^{15}N in the non-N_2-fixing plants or in their growth media. The basic ^{15}N enrichment analysis depends on the mass of N available from three sources and the ^{15}N of each source. The three N sources are those in the atmosphere (lowest ^{15}N), in the soil (moderate ^{15}N) and in the fertiliser (highest ^{15}N). Assuming that the constraining experimental conditions are valid, the contribution of N_2 fixation to whole-plant N can be determined by measuring the ^{15}N of the tested N_2-fixing and non-N_2-fixing reference plants, and the total plant accumulated N.

The following formula can be used to quantify the N_2-fixation if the applied fertiliser is the only available N source to the N_2-fixing plants:

$$N_{fixed} = (1 - atom\%^{15}Nexcess_{N_2\text{-fixing plant}}/atom\%^{15}Nexcess_{fertiliser}) \times N_{N_2\text{-fixing plant}} \quad (1)$$

^{15}N fertiliser can be applied fairly uniformly in an agricultural setting, although significant variation remains. For example, most ^{15}N fertiliser will be near the soil

surface and diluted by soil N; there will be spatial microsite variability and the ^{15}N of soil N decreases with time within the soil profile. Therefore, a multiple-split-block design should be adopted to ensure the accuracy of N_2-fixation in plants if large areas are available for testing (Ledgard *et al.*, 1985). In addition, a non-N_2-fixing reference plant is utilised to compensate for the soil heterogeneity. The reference plant must be selected carefully and planted in an intermixed design with the test plant. Important criteria for reference plant selection include similarity of root zone to the test plant, absence of rhizosphere N_2-fixing organisms, and similarity in seasonal N accumulation. Under those conditions, calculation of N_{fixed} is as follows:

$$N_{fixed} = (1 - \text{atom\%}^{15}\text{Nexcess}_{\text{N2-fixing plant}}/\text{atom\%}^{15}\text{Nexcess}_{\text{non-N2-fixing plant}}) \times N_{\text{N2-fixing plant}} \quad (2)$$

However, the N_2-fixing plant will grow well due to N_2-fixation under low N condition, while growth of the non-N_2-fixing plant may be substantially lower as a consequence of N deficiency. On the other hand, sufficient fertiliser for promoting acceptable growth of the reference plant would reduce N_2-fixation of the N_2-fixing plant. Therefore, the following so-called "*A-value Method*" has been applied to the addition of a higher amount of N to the non-N_2-fixing plant compared with the N_2-fixing plant (Fried and Broeshart, 1975):

$$N_{fixed} = N_{\text{N2-fixing plant}} - \%FR / 100 \times (N_{soil} + N_{fertiliser}) \quad (3)$$

where %FR is the percentage of fertiliser recovery and N_{soil} is the amount of available soil N estimated using the non-N_2-fixing plant.

The ^{15}N enrichment technique has been applied to most agricultural systems over the past 40 years. During the application period, several sources of error have been identified. For example, the application rate of ^{15}N-enriched fertiliser must be low enough so that N_2-fixation is not inhibited by elevated N in the soil. Also denitrification will convert some of the added N fertiliser into N_2 or NO. The most critical source of error comes from selection of the reference plant. If the source ^{15}N for the reference plant is different from that of the test plant, the assay of N_2-fixation is invalidated (Witty and Giller, 1991). All in all, the main weakness of the methodology is that it is too difficult to establish a stable ^{15}N enrichment in the soil

profile in a range of temporal and spatial scales (Danso *et al.*, 1993a). By labeling the growth media with a slow release form of ^{15}N, this shortcoming has been alleviated as shown by Watanabe *et al.* (1990) and McNeill *et al.* (1998).

1.2.3 *^{15}N natural abundance method*

As mentioned above, N_2-fixation in natural communities cannot be assayed with ^{15}N enrichment studies because any addition of N fertiliser would perturb the N balance of the soil and invalidate the measurement of N_2-fixation. Moreover, the added ^{15}N could not be evenly distributed in the soil profile because any mechanism that homogenised the soil profile would severely alter the natural state and modify the root systems. In addition, perennial plants are often present before any fertiliser would be added. The prefertiliser N in plants will mask the effects of ^{15}N accumulated from fertiliser added to the soil. Consequently, the plants contain a large amount of N whose ^{15}N is reflective of prefertiliser conditions. On the other hand, denitrification will convert some of the added N fertiliser into N_2, which may be enriched in ^{15}N compared to the atmosphere because denitrification discriminates against ^{15}N. Meanwhile, N mineralisation discriminates against the heavier isotope. Therefore, in general, soil organic N has greater ^{15}N enrichment than soil inorganic N. Due to these inherent problems in using the ^{15}N enrichment techniques to assay N_2-fixation in natural systems, the natural abundance of ^{15}N has been used more widely for assaying N_2-fixation and N cycling in plant physiological and ecological studies (Handley and Raven, 1992; Nilsen and Orcutt, 1996; Handley and Scrimgeour, 1997; Högberg 1997; Kerley and Jarvis, 1999; Boddey *et al.*, 2000; Tjepkema *et al.*, 2000; Evans, 2001; Robinson, 2001; Unkovich *et al.*, 2001).

It has generally been concluded that the ^{15}N abundance of vegetation reflects the ^{15}N abundance of N sources available to the plants (Shearer and Kohl, 1986). For $\delta^{15}N$, isotope composition can provide information on inputs through N_2-fixation by free living and symbiotic organisms; inputs of fertiliser N; extent of N cycling; sources of N available for plant growth (Nadelhoffer and Fry, 1994; Handley and Scrimgeour, 1997; Högberg, 1997; Evans, 2001; Robinson, 2001; Dawson *et al.*, 2002). The natural abundance method is also dependent on the fact that the soil N pool is

enriched in ^{15}N compared to the atmosphere due to natural processes of the soil N cycle. Furthermore, there must be a large enough difference between soil $\delta^{15}N$ and atmosphere $\delta^{15}N$ (usually about 3 ~ 4 ‰, see Shearer and Kohl, 1986) to measure dilution effects. This technique is similar to soil ^{15}N enrichment experiments because a reference plant is required to account for fractionation due to metabolic processes and to avoid the tedious assay of soil spatial and temporal variation in $\delta^{15}N$.

Natural abundance levels of stable isotopes are expressed as delta (δ) in parts per thousand (‰, per mil). For N (Knowles and Blackburn, 1993):

$$\delta^{15}N\ (‰) = (\text{atom\%}^{15}N_{sample} - \text{atom\%}^{15}N_{air}) / \text{atom\%}^{15}N_{air} \times 1{,}000 \quad (1)$$

where the standard is atmospheric N_2 ($\delta^{15}N$ = zero) by definition (Mariotti, 1983).

The whole plant $\delta^{15}N$ can be calculated by (Handley and Scrimgeour, 1997):

$$\delta^{15}N_{(\text{whole plant})} = [(\delta^{15}N_{(\text{shoot})} \times \text{mg N in shoots}) + (\delta^{15}N_{(\text{roots})} \times \text{mg N in roots})] / (\text{mg N in whole plant}) \quad (2)$$

The assessment of N_2 fixation can be obtained by (Knowles and Blackburn, 1993; Unkovich *et al.*, 1994; Unkovich and Pate, 2001):

$$N_{fixed} = (\delta^{15}N_{\text{non-N2-fixing plant}} - \delta^{15}N_{\text{N2-fixing plant}}) / (\delta^{15}N_{\text{non-N2-fixing plant}} - B) \quad (3)$$

Or: $$\%N_{dfa} = (\delta^{15}N_{\text{non-N2-fixing plant}} - \delta^{15}N_{\text{N2-fixing plant}}) / (\delta^{15}N_{\text{non-N2-fixing plant}} - B) \times 100 \quad (4)$$

where the value "B" refers to the $\delta^{15}N$ value of the effectively nodulated N_2-fixing reference plant grown in media totally lacking external N, and $\%N_{dfa}$ is the percentage of plant N derived from the atmosphere. The measurement relies on the assumption that atmospheric N has a lower ^{15}N abundance than those mineral soil N forms (NH_4^+ or NO_3^-) that are normally available to plants.

Over the past decade or so, the natural abundance technique has been used to evaluate N_2-fixation both in agricultural and natural ecological systems and it has shown a high correlation with N mass balance techniques (Kohl and Shearer, 1980; Shearer and Kohl, 1986; Handley and Raven, 1992; Knowles and Blackburn, 1993;

Handley and Scrimgeour, 1997; Högberg 1997; Boddey *et al.*, 2000; Tjepkema *et al.*, 2000; Robinson, 2001). The greatest advantage of this technique is that it does not perturb those systems prior to any plant harvest. However, when using the $\delta^{15}N$ technique, it should be kept in mind that there is heterogeneity of ^{15}N abundance in natural ecosystems (Hansen *et al.*, 1987; Pate *et al.*, 1993) and that $\delta^{15}N$ values are not identical in all parts of one plant (Shearer and Kohl, 1986). In addition, the selection of an appropriate reference plant always remains difficult (Knowles and Blackburn, 1993; Unkovich *et al.*, 1994, 2001; Unkovich and Pate, 2000, 2001).

1.2.4 *Calculation of nitrogen transfer from N-donor to N-receiver plants*

Estimates of N-transfer from the N_2-fixing N-donor to the non-N_2-fixing N-receiver are based on the assumption that equal proportions of labeled and non-labeled N are transferred. The percentage of total donor-N transferred to the receiver (% $N_{transfer}$) is then estimated from the ratio of labeled N in the N-receiver and total labeled N in N-receiver and N-donor (Ledgard *et al.*, 1985; Ta and Faris, 1987; Giller *et al.*, 1991; Chalk and Smith 1994; Ikram *et al.*, 1994; Jensen, 1996; Johansen and Jensen, 1996):

$$\% N_{transfer} = {}^{15}Ncontent_{N\text{-}receiver} \times 100 / ({}^{15}Ncontent_{N\text{-}receiver} + {}^{15}Ncontent_{N\text{-}donor}) \quad (1)$$

where $${}^{15}Ncontent_{N\text{-}receiver\ or\ N\text{-}donor} = atom\%{}^{15}Nexcess_{N\text{-}receiver\ or\ N\text{-}donor} \times total\ N_{N\text{-}receiver\ or\ N\text{-}donor} / atom\%{}^{15}Nexcess_{labeled\ N} \quad (2)$$

The amount of N (mg plant^{-1}) transferred from the donor ($N_{transfer}$) is calculated as:

$$N_{transfer} = \% N_{transfer} \times total\ N_{N\text{-}donor} / (100 - \%N_{transfer}) \quad (3)$$

The % of N in the receiver derived from transfer (% NDFT) is calculated as:

$$\% NDFT = N_{transfer} \times 100 / total\ N_{N\text{-}receiver} \quad (4)$$

Alternatively, the amount of N transferred may also be calculated by taking into account only the N content in the donor roots, especially in treatments where the donor shoots are harvested. It is then assumed that the N transferred during the growth period had an ^{15}N enrichment equal to the ^{15}N enrichment in the donor roots at the final harvest. Accordingly, the percentage of N in the N-donor roots recovered in the N-receiver plants (% Root $N_{transfer}$) is calculated as:

$$\% \text{ Root } N_{transfer} = \text{Root } N_{transfer} \times 100 / (\text{Root } N_{transfer} + N_{N\text{-donor root}}) \quad (5)$$

where the amount of N (mg plant^{-1}) transferred from the N-donor plant roots (Root $N_{transfer}$) is calculated as:

$$\text{Root } N_{transfer} = \text{Ncontent}_{N\text{-receiver}} \times \text{atom\%}^{15}\text{Nexcess}_{N\text{-receiver}} / \text{atom\%}^{15}\text{Nexcess}_{N\text{-donor root}} \quad (6)$$

By analogy with those equations, it may be possible to use the difference of $\delta^{15}N$ values between plants to determine how much of the N accumulated in root and shoot of a N-receiver plant is derived from the contribution of symbiotically fixed N or other external N by or from a N-donor plant.

1.3 Nitrogen Transfer between Mycorrhizal Plants

The idea that N fixed by a legume may be available to a non-legume present or planted in the same soil originated from studies in the late 1930's (Virtanen *et al.*, 1937; Wilson and Burton 1937). In several of their experiments, these authors found that as much as 10 to 30% of the total N fixed (predominantly in amino acids) in pea was deposited in both sand and soils, and that most of these amino acids were taken up by cereals planted in the same media (Virtanen and Miettinen, 1963). In general, the process of N-deposition and subsequent uptake by another plant is termed N-transfer (Jensen, 1996). Since then, substantial (mostly within a range of 20-50%) N-transfers have been observed in several legume/crop intercropping systems such as soybean or cowpea/maize (*Zea mays*) or sorghum (*Sorghum bicolor*), cowpea/rice (*Oryza sativa*), gram (*Vigna radiata*) or clover/wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) or oat (*Avena sativa*)/vetch (*Vicia sativa*) or lupin (*Lupinus angustifolius*) etc., although some experiments showed little or nearly no N-transfer between them (Fujita *et al.*, 1992; Stern, 1993; Chalk, 1996a, b; 1998).

With or without a split root system, combined with a fine nylon or stainless steel mesh barrier to allow direct mycorrhizal links but not root contact, and using the ^{15}N-isotope labeling technique to label N in the donor plant, many similar observations have been made either through VA mycorrhizae with legume/non-legume plants (van Kessel *et al.*, 1985; Bethlenfalvay *et al.*, 1991; Frey and Schuepp, 1992, 1993;

Johansen and Jensen, 1996) or via ectomycorrhizae with actinorhizal N_2-fixation/non-actinorhizal trees (Arnebrant *et al.*, 1993; Ekblad and Huss-Danell, 1995).

Compared to pure cultivation system, possible benefits may accrue to non-legumes or trees through transfer of N symbiotically fixed by legumes, either through release from nodulated roots, decomposition of dead nodule and root tissue in the soil (Ledgard and Steele, 1992; Tobita *et al.*, 1994; Hogh-Jensen and Schjoerring, 2001; Paynel *et al.*, 2001), mycorrhizal hyphal uptake and translocation (Miller and Allen, 1992; Kapulnik and Douds, 2000), or through common arbuscular mycorrhizal linkages from legumes to grasses (Haystead *et al.*, 1988; Eissenstat, 1990; Bethlenfalvay *et al.*, 1991; Frey and Schuepp, 1992, 1993; Ikram *et al.*, 1994; Johansen and Jensen, 1996). Similar observations have been made through common ectomycorrhizal linkages from the actinorhizal N_2-fixing *Alnus* to non-N_2-fixing *Pinus* (Arnebrant *et al.*, 1993; Ekblad and Huss-Danell, 1995). However, the mechanism of N-transfer and the role of mycorrhizal hyphae in the direct transfer of N are not well established (Smith and Read, 1997). Therefore, studies to identify the pathways of N-transfer and whether mycorrhizal hyphae play a direct role in facilitating N-transfer are needed.

It is difficult to quantify N-transfer accurately because of methodological challenges to exclude N movement with water between plants. ^{15}N can act as a 'tracer' because isotopes allow materials in certain pools (e.g. roots) to be distinguished from those in the rest of an experimental system (e.g. soil). With the ^{15}N dilution method, the medium N pool is labeled with ^{15}N-enriched chemicals or fertilizers and by comparing the enrichment of the N-receiver, an estimate of the transfer of N can be obtained. This method has been used to quantify N-transfer in many intercrops (Cowell *et al.*, 1989; Izaurralde *et al.*, 1992; Waterer *et al.*, 1994; Chalk, 1996a, b; 1998; Unkovich *et al.*, 2000; Paynel *et al.*, 2001). Meanwhile, ^{15}N-depleted chemicals have also occasionally been used to label soil (van Kessel and Roskoski, 1988).

However, its application has some limitations, especially in fertile soil conditions. Firstly, compared to the large volume of soil N, simultaneously available to plants with the ^{15}N-labeled external N, the amount of N transferred from the N-donor to the N-receiver may be much lower than the amount of N taken up from the soil. Secondly, the root depth and pattern of N-uptake of the N-receiver may be different in intercrop and pure system situations, which may cause the ratio of unlabeled-to-labeled N to be different in different growth systems. Both of them can weaken the assumptions for estimating N-transfer.

1.3.1 *Nitrogen nutrition in mycorrhizal plants*

1.3.1.1 *Forms of nitrogen used by mycorrhizal associations*

1.3.1.1.1 *Inorganic N sources*

The uptake and assimilation of inorganic N by mycorrhizal fungi and/or plants is the prerequisite to transfer assimilated N to plants or between plants. The two most readily available inorganic N sources are NO_3^- and NH_4^+ (Haynes and Goh, 1978; Clarkson, 1985; Bloom, 1988; Marschner, 1995; Forde and Clarkson, 1999; Hawkins *et al.*, 2000). Originally, NH_4^+ is mainly derived from the reduction of N_2 symbiotically fixed, while the vast bulk of soil N (organic and inorganic) is initially converted from this sort of reduced-N by a number of microbial processes (Killham, 1994; Paul and Clark, 1996; Brady and Weil, 2002).

In general, NO_3^- is the dominant form of N available to plants and fungi in almost all the aerated agricultural soils due to the rapid nitrification of NH_4^+. In contrast, NH_4^+, which is released from soil humus and other organic N sources by ammonifying organisms, predominates, and NO_3^- may be almost entirely absent, in many undisturbed or very acidic soils (Rice and Pancholy, 1973; Clarkson, 1985; Bloom, 1988; Stewart, 1991; Stewart *et al.*, 1993; Killham, 1994; Paul and Clark, 1996; Brady and Weil, 2002). For conversion of inorganic to organic N, NH_4^+ is directly assimilated by plants, whereas NO_3^- must first be reduced to NH_4^+ in either roots or shoots before it can be assimilated – a process that demands more substantial C and energy costs (Clarkson, 1985; Oaks, 1992; Campbell, 1996). The presence of NH_4^+

often depresses NO_3^- uptake when both occur simultaneously so that many plants seem to take up NH_4^+ preferentially (Marschner *et al.*, 1991; Paul and Clark, 1996; Kreuzwieser *et al.*, 1997; Nordin *et al.*, 2001). In addition, chemically, NO_3^- is highly mobile and is readily transported towards the plant roots by mass flow and diffusion; while NH_4^+ is adsorbed to negatively charged soil particles and transported towards the plant roots mainly by diffusion (Nommik and Vahtras, 1982; Marschner, 1995; Brady and Weil, 2002). As a result, plants require more extensive root systems and/or mycorrhizal symbioses to access NH_4^+ (Raven *et al.*, 1992; Engels *et al.*, 2000).

Mycorrhizal fungi represent an interface between plants and soils, as they mediate the uptake and translocation of elements from the soil particles to the roots of mycorrhizal plants (Allen, 1991; Miller and Allen, 1992; Smith and Read, 1997). AM fungi are generally present in more fertile soils with a higher rate of mineralisation (Sieverding, 1991; Leake and Read, 1997). ECM fungi are abundant in humus, residue and litter layers of top-soil where mineralized N ions are available for their growth and reproduction requirements (Alexander, 1983; Read, 1992; Bending and Read, 1995a). For example, N concentrations in the fungal mat networks of *Hysterangium* and *Gautieria* are significantly higher than that in the attached litter (Entry *et al.*, 1991; Griffiths *et al.*, 1992). This is also true for 24 other species of ECM fungi living in humus but not for those living in litter (Gebauer and Taylor, 1999). In general, NH_4^+ is the preferred N source rather than NO_3^- for most mycorrhizal fungi (Abuzinadah and Read, 1988; Finlay *et al.*, 1992; Keller, 1996; Baar *et al.*, 1997, Sarjala, 1999; Putra *et al.*, 1999). There are too many reports to cite here that show mycorrhizal hyphae can capture inorganic NH_4^+ and/or NO_3^- from the growth media and then translocate N into the mycorrhizal roots of partner plants, or transfer N between plants (Smith and Read, 1997; Varma and Hock, 1998; Kapulnik and Douds 2000; Hawkins *et al.*, 2000). The following references have described the uptake kinetics of inorganic N (Littke *et al.*, 1984; Jongbloed *et al.*, 1991; Plassard *et al.*, 1994; Eltrop and Marschner, 1996; Gessler *et al.*, 1998; Wallenda *et al.*, 2000) and of amino acids (Jones and Darrah, 1994; Chalot *et al.*, 1995, 1996; Wallenda and Read, 1999; Wallenda *et al.*, 2000; Nasholm and Persson, 2001) by mycorrhizal fungi and/or roots.

Assimilation of NH_4^+ in mycorrhizal fungi or plants has also been studied following its absorption. NH_4^+, either directly absorbed by mycelia, or indirectly derived from the reduction of NO_3^-, is rapidly assimilated into glutamate and glutamine in mycorrhizal roots, in which the assimilation pathways involving GS (glutamine synthetase), GOGAT (glutamate synthetase) and GDH (glutamate dehydrogenase) are all present (France and Reid, 1983; Smith *et al.*, 1985; Cliquet and Stewart, 1993; Martin and Botton, 1993; Botton and Chalot, 1998; Johansen *et al.*, 1996; Martin and Plassard, 2001). Glutamate and glutamine are then used to synthesise other amino acids such as alanine, γ-aminobutyrate etc. within the foraging hyphae. In general, the major products are glutamate and glutamine, with alanine, arginine, and aspartate-asparagine also important in ECMs (Finlay *et al.*, 1992; Martin and Botton, 1993; Ek *et al.*, 1994; Botton and Chalot, 1998; Martin and Plassard, 2001). A conceptual model of three basic patterns of N incorporation has been as follows in different types of ectomycorrhizae: (1) GS occurs in the fungal sheath while GOGAT is in the mycorrhizal root in *Fagus*; (2) both GS and GDH exist in the sheath and the extraradical mycelium of *Picea*, respectively; (3) the GS-GOGAT pathway operates in the mycorrhizae formed by *Pisolithus tinctorius* (Martin and Bottton 1993; Martin and Plassard, 2001). In addition, NH_4^+ assimilation occurs through the GS-GOGAT pathway in *Fagus-Laccaria* ECMs, but through NADP-GDH and GS in *Abies-Hebeloma* ECMs (Botton and Chalot, 1998). On the other hand, NH_4^+ is metabolised via the GS-GOGAT pathway in AM associations (Cliquet and Stewart, 1993; Johansen *et al.*, 1996). Assimilated-N compounds (amino acids and amides) are ready for transport across the fungus-root interface and distribution or transfer from root to shoot, or to neighbouring plants (see Smith and Read, 1997).

1.3.1.1.2 *Organic N sources*

Many ECM fungi have the ability to use organic N sources (Smith and Read, 1997; Nasholm *et al.*, 1998; Wallenda and Read, 1999; Lipson and Nasholm 2001; Nasholm and Jersson, 2001), either in the forms of amino acids (Abuzinadah and Read, 1988; Chalot *et al.*, 1994a, b; Kielland, 1994; Turnbull et al., 1995; Chalot and Brun, 1998; Dickie *et al.*, 1998; Nasholm *et al.*, 1998, 2000; Tibbett *et al.*, 1998; Chen *et al.*, 1999; Lipson *et al.*, 1999; Nehls *et al.*, 1999; Wallenda and Read, 1999;

Anderson *et al.*, 2001), urea (Yamanaka, 1999; Nakano *et al.*, 2001a, b), peptides or proteins due to their proteolytic capacities (Abuzinadah and Read, 1986a, b; Abuzinadah and Read, 1989; Finlay *et al.*, 1992; Keller, 1996; Baar *et al.*, 1997; Leake and Read, 1997), or directly mobilise N from organic materials (Entry *et al.*, 1991; Bending and Read, 1995a, b; Perez-Moreno and Read, 2000) in addition to NO_3^- and NH_4^+. Referred to as "protein fungi", *Amanita muscaria, Cenococcum geophilum, Paxillus innolutus, Rhizopogon roseolus, Suillus bovinus* and *Hebeloma crustuliniforme* can use bovine serum albumin, gelatin, gliadin and other peptides, from di- to penta-peptides. In contrast, *Laccaria laccata* and *Lactarius rufus* are called "non-protein fungi" because they cannot use protein. The most common ectomycorrhizal fungus, *Pisolithus tinctorius*, has a limited ability to use protein for growth.

It is generally thought that AM fungi do not possess degradative ability and are unable to capture nutrients from complex organic materials. However, uptake of aspartic acid and serine had been found in AM fungus *Glomus fasciculatum* inoculated *Lolium perenne* (ryegrass) (Cliquet *et al.*, 1997) and glycine in AM grass *Deschampsia flexuosa* (Wavy Hair-grass) (Nasholm *et al.*, 1998). In contrast, *Glomus mosseae* did not enhance N exploitation or affect the form in which N was captured by *Plantago lanceolata* (plantain) from ryegrass shoot material (Hodge *et al.*, 2000). Hodge (2001) also indicated that the presence of three AM fungi (*G. mosseae*, *G. hoi* and *Scutellospora dipurpurescens*) did influence the decomposition of, but not N capture from, glycine patches in soil. However, more recently Hodge *et al.* (2001) demonstrated that AM fungus *G. hoi* can accelerate decomposition and acquire N directly from an organic material of ryegrass shoots, although the mechanism is not known. Whether or not other AM fungi have similar functions remains to be established.

1.3.1.2 *Mycorrhizal effects on nodulation and N_2-fixation*

Frank first suggested the possibility of a direct involvement of ectomycorrhizal fungi in N acquisition by plants in 1894 (see Smith and Read, 1997). 50 years after Frank's work, the probably first observation of growth, nodulation and mycorrhizal status on a large number of legumes was made by Asai in 1944 (see Smith and Read, 1997).

Since then, nodulation and N_2 fixation by mycorrhizal and non-mycorrhizal plants have been the subjects of a number of experiments (Rose 1980; Rose and Youngberg, 1981; Barea et al., 1987; Diem and Dommergues, 1990; Arnebrant *et al.*, 1993; Ekblad and Huss-Danell, 1995; Martin *et al.*, 1995; Binkley and Giardina, 1997; Wheeler *et al.*, 2000). In most cases, improved nodulation and N_2 fixation in mycorrhizal plants appears to be the result of relief from P stress and possibly enhanced uptake of some other essential micronutrients, resulting in general improvement in growth and yield and indirect effects upon the N_2-fixing system. The differences between mycorrhizal and non-mycorrhizal plants usually disappear if the latter are supplied with a readily available P source (Barea *et al.*, 1989; Azcon and Barea, 1992; Bethlenfalvay, 1992). Other reports also indicated that the mycorrhizal effect on root N uptake depends on the form of inorganic N (NH_4^+ or NO_3^-) and the mycorrhizal type (AM or ECM) (Wallander *et al.*, 1999; Constable *et al.*, 2001).

As mentioned above, the two major forms of plant available N source in soil are NO_3^- and NH_4^+. NO_3^- is highly mobile and is readily transported towards the plant roots by mass flow and diffusion. NH_4^+ is adsorbed to negatively charged soil particles and transported towards the plant roots mainly by diffusion (Nommik and Vahtras, 1982; Brady and Weil, 2002). It was therefore assumed that mycorrhizal hyphae might contribute more to the transport of NH_4^+ than NO_3^- towards plant roots. In this way, N was thought to move from the N-donor to the N-receiver through the hyphae without entering the soil solution, the N-concentration difference in the two plants constituting the driving force in the translocation (Frey and Schuepp, 1993). However, substantial N-transfer from donor to receiver took place only when the donor was supplied with mineral N but not when relying on N_2 fixation (Bethlenfalvay *et al.*, 1991). It is not known, whether N-transfer is restricted to the plant-fungal tissues, whether net transfer of N from one plant to another occurs only unidirectionally, or whether N is translocated between plants in both directions (which would decrease net N transfer). Therefore, studies are needed in which different inorganic external $^{15}NH_4^+$ and $^{15}NO_3^-$ sources are applied to investigate whether there is a net transfer of N from one plant to another via mycorrhizal hyphae and *vice versa*.

1.3.2 *Common mycorrhizal networks*

Plants usually grow naturally close together, either in a single-species population but mostly in multiple-species communities. Most plants, especially grasses and trees, hide almost half their biomass in an enormous mingle of roots in the below-ground soils. Mycorrhizal fungi are ubiquitous components of most soil ecosystems. They grow through the soil, colonise the roots of various plants and are capable of forming links between plant species (Newman, 1988; Newman *et al.*, 1992). Because of little host specificity in colonisation (Smith and Read, 1997), plant roots are linked by a common mycorrhizal network (CMN) of either AM or ECM fungi, forming 'the wood-wide-web', a term coined by the prestigious scientific journal ***Nature*** (see the cover of Volume 388, 7 August 1997). These, in turn, are usually woven into an even bigger tangle of fungi and roots (Newman, 1988).

Mycorrhizal links between plants have been shown by direct eye-view visual observation (Heap and Newman, 1980a, b; Francis and Read, 1984; Finlay and Read, 1986a, b; Newman, 1988; Newman *et al.*, 1992; 1994) and by isotope autoradiography (Hirrel and Gerdemann, 1979; Chiariello *et al.*, 1982; Francis and Read, 1984; Read *et al.*, 1985; Finlay and Read, 1986a; Read, 1991; McKendrick *et al.*, 2000; Wu *et al.*, 2000; Lerat *et al.*, 2002). There is also indirect evidence that plants from the same population are able to share a more efficient hyphal network (Ronsheim and Anderson, 2001; Onguene and Kuyper, 2002). One possible consequence of the existence of these AM or ECM links or networks is to provide a pathway for nutrient movement between adjacent plants. Groups of plant species joined together in this way have been known as functional guilds (Perry *et al.*, 1989), which facilitate nutrient uptake and translocation (Read, 1997). Nutrients such as C (Bjorkman, 1960; Reid and Woods, 1969; Hirrel and Gerdemann, 1979; Brownlee *et al.*, 1983; Francis and Read, 1984; Finlay and Read, 1986a; Grime *et al.*, 1987; Duddridge *et al.*, 1988; Martins, 1992, 1993; Waters and Borowicz, 1994; Ek *et al.*, 1996; Watkins *et al.*, 1996; Graves *et al.*, 1997; Simard *et al.*, 1997a, b, c; Fitter *et al.*, 1998; McKendrick *et al.*, 2000; Wu *et al.*, 2001), N (van Kessel *et al.*, 1985; Haystead *et al.*, 1988; Eissenstat, 1990; Bethlenfalvay *et al.*, 1991; Frey and Schuepp, 1992, 1993; Arnebrant *et al.*, 1993; Ikram *et al.*, 1994; Ekblad and Huss-Danell, 1995; Ek *et al.*, 1996; Johansen and Jensen, 1996) and P (Heap and Newman,

1980b; Chiariello *et al.*, 1982; Whittingham and Read, 1982; Finlay and Read, 1986b; Newman and Ritz, 1986; Eissenstat, 1990; Eason *et al.*, 1991; Newman and Eason, 1993; Tuffen *et al.*; 2002) might then move through the CMN, from plant to plant.

1.3.2.1 *Nitrogen transfer between arbuscular mycorrhizal plants*

As early as 1976, Hains and Best showed that loss of NH_4^+ and NO_3^- from soil by leaching with water was retarded when plants of *Liquidambar styraciflua* (sweetgum) were infected with the mycorrhizal fungus, *Glomus mosseae*. Unfortunately, the mycorrhizal plant root systems were considerably larger than the non-mycorrhizal ones, so that the results did not unequivocally indicate that the mycorrhizal fungi themselves were involved. Subsequently, Ames *et al.* (1983) found that when organic ^{15}N was used on extraradical hyphae of mycorrhizal celery (*Apium graveolens)*, N-transfer took a considerable period of time to occur. They assumed that mineralisation by soil microflora was an essential step in making the organic N available and that this caused the delay. By using a mesh-compartmented system, Ames *et al.* (1983) found that more inorganic $(^{15}NH_4)_2SO_4$ applied to the hyphal compartment (HC) was transported into mycorrhizal celery plants than into non-mycorrhizal plants. The amount was correlated with the percentage colonisation of the roots, with the hyphal length density in the HC and with the number of hyphal crossing of the mesh. By using a split-root system, a range of 10-20% of ^{15}N-transfer from soybean to maize (van Kessel *et al.*, 1985; Bethlenfalvay *et al.*, 1991), from subterranean clover (*Trifolium subterraneum*) to ryegrass (*Lolium rigidum*) (Haystead *et al.*, 1988) and from lucerne (*Medicago sativa*) to ryegrass (Barea *et al.*, 1989) was observed, and the transfer rate was significantly increased when the plants were mycorrhizal. Bethlenfalvay *et al.* (1991) also found that N transfer from neighbouring soybean to maize may be driven by a source-to-sink relationship. By growing two seedlings of *P. lanceolata* in a three-pot unit in which each of their root systems were split, with part in the central shared pot and part by themselves in an outside pot, Eissenstat (1990) also found ^{15}N transfer from donor to receiver was about 10-fold higher than ^{32}P transfer and in amounts that could potentially affect the receiver nutrition in nutrient-deficient soils.

When $^{15}NH_4$ was supplied to HCs formed by *Glomus intraradices,* the results showed hyphal ^{15}N translocation and transfer to mycorrhizal cucumber (*Cucumis sativus*) and the ^{15}N in the HC was significantly depleted (Johansen *et al.,* 1992; Johansen *et al.,* 1993a, b). However, none of these hyphal N translocations and transfer resulted in an increase in plant N content or growth. Johansen *et al.* (1992) suggested that the small physical size of the experimental systems might have contributed to this. They proposed that where soil N is present as NH_4^+, or where strong competition exists for recently mineralised N, mycorrhizal mycelia might play a significant role in N acquisition. Differences between fungi species in accessing ^{15}N were also related to differences in hyphae distribution in the HC (Frey and Schuepp, 1992). Additional evidence for the competitive effects of mycorrhizae in accessing less available forms of N was obtained by using ^{15}N dilution methods (Azcon-Aguilar *et al.*, 1993; Tobar *et al.*, 1994a). More recently, using a split-root system, Johansen and Jensen (1996) demonstrated that 15% of the donor pea root N was transferred to the receiver barley, when plants were colonised by *G. intraradices*. However, all of those studies except for Bethlenfalvay *et al.* (1991) were carried out under conditions in which the root systems were not separated by an interposed compartment to avoid direct root contact between N_2-fixing and non-N_2-fixing plants.

There have been only six studies reported to date of mycorrhizal effects with ^{15}N-labeled NO_3^- (Frey and Schuepp, 1993; Johansen *et al.,* 1993b; Ikram *et al.*, 1994; Tobar *et al.*, 1994b; Martin *et al.,* 1995; Martensson *et al.,* 1998). Using the split-root system, Frey and Schuepp (1993) found only 0.1% of the ^{15}N transferred from *Trifolium alexandrinum* (berseem) to maize when $K^{15}NO_3$ solution was injected into the root. However, 4.7% ^{15}N was transferred from berseem to *Malus* × *domestica* Borkh (apple) when $^{15}NO_3^-$ was injected into the leaf petioles of berseem. Applying ^{15}N at 5cm distance from the root compartment, Johansen *et al.* (1993) showed that the recovery of ^{15}N from both $^{15}NH_4^+$ and $^{15}NO_3^-$ supplied to the hyphal compartment was 38% and 40% respectively, when cucumber roots were colonised by *G. intraradices*, compared to 7% and 16% in non-mycorrhizal cucumber roots. In addition, the external hyphae reduced the level of mineral soil N of the mycorrhizal hyphal compartments compared to non-mycorrhizal controls. They indicated that ^{15}N

variations in both plant and soil suggested that VA hyphae transported both $^{15}NH_4^+$ and $^{15}NO_3^-$ to the host plants. But under well-watered conditions and using mesh-compartmented pots as well, no difference in $^{15}NO_3^-$ enrichment between mycorrhizal (*G. fasciculatum*) and non-mycorrhizal plants of lettuce (*Lactuca sativa*) was observed. This was similar to the $^{15}NO_3^-$ transfer observation from puero (*Pueraria phaseoloides*) to rubber (*Hevea brasiliensis*) via hyphal links of the same AM fungus *G. fasciculatum* (Ikram *et al.*, 1994). However, in dry soil, the $^{15}NO_3^-$ level of the mycorrhizal (*G. fasciculatum*) plants was four times higher, probably reflecting the much lower mobility of NO_3^- in dry soil (Tobar *et al.*, 1994b). They suggested that arbuscular mycorrhiza could be important for plant N-nutrition in dry soil where NO_3^- is the major N source. Martin *et al.* (1995) reported that $^{15}NO_3^-$ transfer from greenhouse grown soybean to maize via the AM fungus *G. intraradix* did occur at moderate P, low N and in full light, and at low P, high N and in shade conditions. This indicated that differences in relative competitiveness for mineral N, N partitioning among plant organs, and ^{15}N uptake were not affected by P or N availability. In one experiment, Martensson *et al.* (1998) found that 3 to 50% of N in chicory (*Cichorium intybus*) was derived from pea. In another experiment, ~15%, ~60% and ~25% of N in 3, 4.5 and 6 months old chicory came from red clover (*Trifolium pratense*). They suggested that there is potential for improving N-transfer in intercropping systems through the methodological selection of suitable plant and mycorrhizal partners.

Arbuscular mycorrhizal effects on N-transfer have also been studied under field conditions. When intercropped within the same rows an increase from 45% to twofold in ^{15}N-transfer between soybean and maize was observed in mycorrhizal plots, along with a relative increase in maize productivity (Hamel and Smith, 1991, Hamel *et al.*, 1991; Hamel and Smith, 1992). This suggests that mycorrhizal fungi may be involved in N redistribution in a plant community. However, the results on N-transfer between field-grown plants were somewhat inconclusive. The same authors did not find any direct transfer of fixed N from soybean to maize via AM hyphae when intercropped in alternative rows (Hamel *et al.*, 1991; Hamel and Smith, 1992). Close contact between plant-root systems has been suggested as a necessary condition for N-transfer by AM. Hamel *et al.* (1992) even showed a lower ^{15}N

enrichment trend of the grasses grown in mycorrhizal plots in AM lucerne/grass mixtures.

1.3.2.2 *Nitrogen transfer between ectomycorrhizal plants*

In general, the evidence for direct transport of inorganic nutrients via mycelial connections is stronger for arbuscular mycorrhizal plants than for ectomycorrhizal plants (Arnebrant *et al.*, 1993; Marschner and Dell, 1994). Melin and Nilsson (1950, 1953) were probably the first to demonstrate that hyphae of ECM plants were able to take up N and transfer it to roots. Since then, there have been a number of reports of inorganic N uptake and translocation from media to roots via vegetative ECM fungi (Miller and Allen, 1992; Marschner and Dell, 1994; Smith and Read, 1997). ECM fungi and roots can exploit organic N, which is normally unavailable to AM fungi or to non-mycorrhizal roots. Significant N-transfer is therefore more likely between N_2-fixing and non-N_2-fixing plants when they are both ectomycorrhizal than when both are AM mycorrhizal. Arnebrant *et al.* (1993) found that 5% to 15% of the ^{15}N in *Pinus contorta* was transferred from symbiotically fixed N by alder (*Alnus glutinosa*) when they were connected by a common ectomycorrhizal mycelium. In addition, Ekblad and Huss-Danell (1995) indicated that the proportion of fixed N in *Pinus sylvestris*, transferred from *Alnus incana*, was highest (9.0%) when *Pinus* was N starved and ectomycorrhizal, and the N_2-fixation of *A. incana* was maximal as well. However, they concluded that there was then no support for a mycorrhiza mediated N-transfer between living trees great enough to improve growth of the N-receiver.

1.3.3 *Application of ^{15}N natural abundance to investigate N transfer between plants*

1.3.3.1 $\delta^{15}N$ values in mycorrhizal plants

The comparison reported by Högberg (1990) indicated that there was a significant difference of 1.0~2.5‰ $\delta^{15}N$ between ECM and AM species, and between ECM and nodulated non-mycorrhizal plants in the Miombo woodlands of Tanzania. These higher $\delta^{15}N$ patterns in ECM plants were not confirmed in Cameroon's humid forests (Högberg and Alexander, 1995) nor in subarctic regions of Northern Sweden (Michelsen *et al.*, 1996), although $\delta^{15}N$ values in N_2-fixing plants was almost 4.0‰ above that of atmospheric N_2. This might account for the variability of plant $\delta^{15}N$

with different mycorrhizal status and life form found in a *Banksia* woodland (Pate *et al,* 1993). They found that non-mycorrhizal plants were ~2.0‰ more ^{15}N-enriched than either AM, ECM/AM or ericoid ones in such an ecosystem, most likely because of higher NO_3^- uptake by the non-mycorrhizal plants. A similar $\delta^{15}N$ pattern among plant species in a subtropical wet *Wallum* heathland suggested no differential NO_3^- uptake (Schmidt and Stewart, 1997). $\delta^{15}N$ values also were higher in non-AM than in AM *Ricinus communis*, *Acacia* and *Sorghum* (Handley *et al.,* 1993; Michelsen and Sprent, 1994; Fonseca *et al.*, 2001), but there was no difference in $\delta^{15}N$ values between non-ECM and ECM *Eucalyptus* (*E. globulus*) or pine (*P. sylvestris*) trees when only inorganic N was used (Handley *et al.,* 1993; Högberg *et al.*, 1994).

Significant leaf $\delta^{15}N$ differences were found in the tundra heath-boreal forest transition zone of Alaska (Schulze *et al.* 1994): *Picea mariana* (–6.50‰) < *Vaccinium vitisidaea* (–3.84‰) < *Calamagostis canadensis* (+0.59‰). Distinctive rooting depths may have contributed to these but isotope discrimination, facilitated by either ECMs or AMs, is also a possibility. Support for this has been obtained in field-based studies, in which the extent and type of mycorrhizal infection was examined in the fellfield and heathland communities (Michelsen *et al.,* 1996). In the fellfield, the mean $\delta^{15}N$ values were –5.5‰ for ericoid mycorrhizal species, –4.1‰ for ECM species and 0 for AM or non-mycorrhizal species. In the heathland for the same mycorrhizal groups, $\delta^{15}N$ values were –7.6, –6.4 and –1.8‰, respectively. In all cases $\delta^{15}N$ values in the ECM plants were significantly different from those in the AM or in the non-mycorrhizal species. Also $\delta^{15}N$ values in ECM or ericoid mycorrhizal plants (56 plant and 6 fungus species) were 3.5 – 7.7‰ lower than their non-mycorrhizal counterparts in health and forest tundra ecosystems in Greenland, Siberia and Sweden (Michelsen *et al.,* 1998). In summary, Michelsen *et al.* (1998) thus proposed a general $\delta^{15}N$ pattern: non-mycorrhizal/AM > ECM ≥ ericoid mycorrhizal plants in ecosystems with nutrient-deficient organogenic soils.

Schmidt and Stewart (1997) reported leaf $\delta^{15}N$ values of 39 species in a subtropical heathland in Australia. The group average $\delta^{15}N$ values of non-mycorrhizal, N_2-fixing and mycorrhizal (ECM/AM, AM or Ericoid) species were 1.8‰ (0.3‰ – 4.3‰, highest), –1.6‰ (2.4‰ – –0.5‰) and –3.4‰ (–6.3‰ – –1.8‰, lowest),

respectively. The range of $\delta^{15}N$ values from –6.3‰ to 4.3‰ showed consistent differences between mycorrhizal and non-mycorrhizal species, indicating non-mycorrhizal species were significantly ^{15}N-enriched but mycorrhizal ones strongly ^{15}N-depleted. They proposed that there was discrimination against ^{15}N during transfer of N from the fungus to the plant.

In addition, $\delta^{15}N$ values were 5-10‰ higher in the sporocarps of ECM fungi than that of their alleged host plants (Gebauer and Dietrich, 1993; Handley *et al.* 1996; Taylor *et al.*, 1997), 2.4-6.4‰ enriched in the fungal sheaths of ECMs than in the remaining root-core materials (Högberg *et al.*, 1996), and 2.8–4.9‰ higher in fungal rhizomorphs than in the plant materials (Högberg *et al.*, 1999). These results indicated that ^{15}N fractionation might occur during transfer of assimilated N from the ECM sheath to the root core, resulting in ^{15}N enrichments in ECM root tips and/or fruit bodies of ECM fungi. However, mass balance calculation found that plant $\delta^{15}N$ was only –0.1‰ decreased because a high level efficiency of N-transfer ensured that almost all the acquired-N by the fungi was translocated into the plants. Three possible explanations for this different $\delta^{15}N$ pattern were thus proposed: (1) uptake of N sources with different $\delta^{15}N$ values; (2) differences in fractionation during uptake; (3) host plants and mycorrhizal symbionts having different physiologies.

Meanwhile, amino acid biosynthesis often resulted in a lower $\delta^{15}N$ in amino acids than in their precursors. For example, transamination of glutamic to aspartic acid caused a 9.0‰ $\delta^{15}N$ lower than that of the source (Macko *et al.*, 1986). Consequently, translocation of amino acids with a relatively lower $\delta^{15}N$ to the host plant would cause the plant to have a lower $\delta^{15}N$, and the fungi a greater $\delta^{15}N$, than the N source used (Evans, 2001). Because the biomass of the fungi was much less than that of the plant, therefore, the effect of the mycorrhizae would be negligible, so that plant $\delta^{15}N$ would reflect the N source used (Evans, 2001). However, Emmerton *et al.* (2001a, b) showed that although $\delta^{15}N$ values of mycorrhizal fungi were significantly shifted during N uptake and metabolism, and that plant $\delta^{15}N$ values always differed from the N-sources and were also influenced by ECM, plant N source could not be identified by $\delta^{15}N$ values of ECM plants. This is also the case in AM barley and lettuce seedlings (Azcon-G.-Aguilar *et al.*, 1998).

It has long been evident that plant $\delta^{15}N$ values mainly mirrors the processes which fractionate $^{15}N/^{14}N$ rather than the $\delta^{15}N$ of plant N source(s), because most of the observed fungal ^{15}N enrichment is due to massive protein turnover in the vegetative mycelia and subsequent loss of NH_4^+–N to the growth medium or soil, suggesting that the fungal $\delta^{15}N$ was indicative of organic N used by the fungus and this might further indicate a transmittance of ^{15}N-depleted N to associated plants (Gebauer and Dietrich, 1993). However, the benefit extent of host plants from various soil organic N through mycorrhizal fungi has not been established, although it is clear that mycelia of mycorrhizal fungi transfer N from the soil to plant roots (Smith and Read, 1997). The $\delta^{15}N$ of soil NH_4^+ remained relatively constant, and was similar to foliar $\delta^{15}N$ values on sites with the highest N availability. In contrast, foliar $\delta^{15}N$ values were depleted by 2.0-6.0‰, and mycorrhizal fungi enriched by 4.0-6.0‰, compared with soil NH_4^+ on sites with low N availability (Hobbie *et al.*, 2000). Both field and modeling studies suggested that plants would require little N from mycorrhizae when the soil inorganic N was abundant (Hobbie *et al.*, 1999a, b; 2000). They concluded plants took up NH_4^+ when availability was high, but mainly relied on mycorrhizal fungi when NH_4^+ availability was low. Fractionation during mycorrhizal transfer of N provided the best explanation for patterns observed in the field.

1.3.3.2 Application of $\delta^{15}N$ to trace N-transfer

Previous studies have shown that host plants and mycorrhizal associates differed in their $\delta^{15}N$ values by as much as ~10‰ (Högberg, 1997; Handley and Scrimgeour, 1997; Handley *et al.*, 1998; Evans, 2001; Robinson, 2001) and became more negative as nutrients turned more insufficient (Hobbie *et al.*, 1999a, b), suggesting that the effects on plant $\delta^{15}N$ of ECM and ericoid mycorrhizae could account for a large part (~25%) of the variation in range of foliar $\delta^{15}N$ (Handley *et al.*, 1999). $\delta^{15}N$ values could also provide information about the possible influence of mycorrhizal fungi or mycorrhization on changes in plant N status in response to different external N supplements, as $\delta^{15}N$ was generally depleted relative to that of available N (Schmidt and George, 1997; Hobbie *et al.*, 1999a, b; 2000). The most plausible mechanism to explain low $\delta^{15}N$ values in plant foliage was a large isotopic

fractionation (8–10‰) during transfer of N from mycorrhizal fungi to host plants (Hobbie *et al.*, 2000). They suggested that the correlation of foliar $\delta^{15}N$ and N concentration reflected the changes in the fractionation of N obtained through mycorrhizae, or in the proportion of mycorrhizal N transferred to foliage, or both.

In addition, inorganic ^{15}N uptake in the field appeared to occur predominantly through the extramatrical hyphae, but not through either mycorrhizal or non-mycorrhizal roots (Wallenda *et al.*, 2000). This was supported by a recent $\delta^{15}N$ study that direct root N absorption was not an important factor in N uptake (Hobbie *et al.*, 2001). Organic N could be also used by mycorrhizal fungi (Smith and Read, 1997; Nasholm, 1998; Martin *et al.*, 2001; Martin and Plassard, 2001; Nasholm and Persson, 2001). Another pathway is through the internal cycling of fungal N (Handley *et al.*, 1996). Meanwhile, the lack of a mycorrhiza, or variation in the species of AM-forming fungal associations, could lead to plant ^{15}N enrichment, rather than depletion (Handley *et al.*, 1999). All these factors would affect $\delta^{15}N$ values of both fungi and plants.

$\delta^{15}N$ values provide insight into interactions between mycorrhizal fungi and plants (Michelsen *et al.*, 1998; Hobbie *et al.*, 1999a, Högberg *et al.*, 1999; Hobbie, 2000). Depending on nutrient availability, the presence or absence and type of mycorrhiza could have effects on plant $\delta^{15}N$ values. The range of $\delta^{15}N$ values increased with external nutrient deficiency or became statistically indistinguishable under nutrient sufficient conditions (Hobbie *et al.*, 1999a, b; Chang and Handley, 2000). A general 5-10‰ of foliar $\delta^{15}N$ difference was related to mycorrhizal types: non-mycorrhizal/AM > ectomycorrhizal > ericoid (Michelsen *et al.*, 1998). These patterns indicated that $\delta^{15}N$ values or fractionations could be used to trace N exchange between plants with different N requirement and mycorrhizal status.

Since the transfer of isotopically depleted N from either ECM or AM fungi to host plants has been observed by many researchers (Schmidt and Stewart, 1997; Hobbie *et al.*, 1999a, b; Hobbie *et al.*, 2000; Hobbie *et al.*, 2001), it is possible to use $\delta^{15}N$ values to evaluate how much of the N can be transferred from one plant to another. However, $\delta^{15}N$ can be used as an indicator or parameter to trace mycorrhiza-

mediated N exchange between plants only if differences in $\delta^{15}N$ are distinguishably and verifiably large between plants.

1.3.3.3 Use of $\delta^{15}N$ for N-transfer investigations

As described in Section 1.2.3, $\delta^{15}N$ has been extensively applied in N_2-fixation studies in agricultural and natural ecological systems, with a comparable precision with both N mass balance and ^{15}N enrichment techniques. It is a surprise that ^{15}N natural abundance has not been more widely employed, or at least used as much as enriched ^{15}N, in N-transfer studies, due to the mentioned problems in Section 1.2.3 of this Chapter. Another reason may be that the interpretation of isotopic compositions in mycorrhizal fungi and mycorrhizae is complex, particularly when there exists a bidirectional N transport between fungi and plants (Martin and Botton, 1993). The questions are whether the $\delta^{15}N$ natural abundance is reliable and the $\delta^{15}N$ values can be employed to investigate N-transfer between either ECM or AM plants.

The first effort to use $\delta^{15}N$ to investigate N-transfer between non-mycorrhizal plants was reported by Binkley *et al.* (1985). They measured $\delta^{15}N$ values of soil N and foliage N from Douglas-fir (*Pseudotsuga menziesii)*, and Sitka alder in mixed conifer/alder stands at four locations in the Pacific Northwest of North America. Although soil NH_4^+ was significantly depleted in ^{15}N and $^{15}N/^{14}N$ ratios differed significantly among all five stands, there were no consistent patterns. They concluded that isotopic discrimination against ^{15}N did occur during N transfer at these sites, but suggested that the $\delta^{15}N$ technique could not provide a simple means for investigating N cycling in natural ecosystem. Kohls *et al.* (1994) found there was no isotopic change in non-N_2-fixing plants growing within 1 m of non-nodulated, actinorhizal *Dryas* plants. However, the leaf $\delta^{15}N$ values were closer to atmospheric N_2 in the non-N_2-fixing plants growing within 1 m of nodulated *Dryas*, which showed active N_2-fixation ability. This indicated that some symbiotically fixed-N had translocated from the N_2-fixing plants to the non-N_2-fixing ones. van Kessel *et al.* (1994) also reported rapid N cycling through N-mineralisation contributed from the N_2-fixing plant to the non-N_2-fixing one. Compared to N_2-fixing *Leucaena leucocephala*, the non-N_2-fixing understorey species had a significant enrichment in ^{15}N. In addition, $\delta^{15}N$ in the understorey vegetation decreased significantly and was

almost identical to that of older *L. leucocephala.* The results indicated that a decline over time in $\delta^{15}N$ of understorey vegetation under N_2-fixing *L. leucocephala* shrubs was evidence of a transfer of fixed-N. The $\delta^{15}N$ variations in understorey species could also be attributed to net N-mineralisation of shedding biomass and root exudation from *L. leucocephala.* That is, a portion of the symbiotically fixed-N was made available through the decomposition and subsequent incorporation into the available soil-N pool from the shed *L. leucocephala* biomass to the understorey species. They claimed it was the first direct evidence of applying the ^{15}N natural abundance technique to trace internal N cycling between an N_2-fixing tree and its non-N_2-fixing understorey vegetation within an agroecosystem. Unfortunately, these three studies did not check whether mycorrhizal colonisation had an effect on the $\delta^{15}N$ variations in all the stands where they were sampled.

1.4 Rationale for This Study

It was known that cereals intercropped with legumes generally benefit from the association in terms of increased grain and N yields per unit area compared with monocropped cereals (Fujita *et al.*, 1992; Stern, 1993; Chalk, 1996a, b; 1998; Ledgard, 2001). This indicates that N-transfer from an N_2-fixing plants (N-donor) to an associated non-N_2-fixing plant (N-receiver) is most likely to occur in nature through either the interception and uptake of released fixed-N from the N-donor by the roots of the N-receiver, or via the root mycorrhizal link (Newman, 1988; Newman *et al.*, 1992) between N-donor and N-receiver plants (Marschner and Dell, 1994; Smith and Read, 1997). Therefore, in both agricultural and natural ecological communities, mycorrhizal associations may be important factors influencing the performance of both N-donor and N-receiver plants through the acquisition and translocation of N by the mycorrhizal fungus, particularly when the relatively immobile NH_4^+ rather than the mobile NO_3^- is the major source of plant available N.

N_2-fixing and non-N_2-fixing plants can provide a good example for investigating the N-transfer between the plants where neighbouring plants may have differing N status. In general, with or without a split root system, combined with the fine nylon or stainless steel mesh to allow the direct mycorrhizal link but not root contact,

together with ^{15}N-isotope labeling method to enrich the N in the N-donor plant, some experiments have demonstrated a below-ground N-transfer whereas others found no evidence for transfer of N between N-donor and N-receiver plants via mycorrhizal hyphae (see Section 1.3 this Chapter). Furthermore, there is controversy about the extent to which direct N-transfer is actually being facilitated by mycorrhizal hyphae or by other indirect means such as soil pathways, and whether it is of agricultural or ecological significance if N-transfer does occur between these plants through mycorrhizal hyphae (Newman, 1988; Newman *et al.*, 1992; Tobita *et al.*, 1994).

In addition, differences in N nutritional requirements between donor and recipient may result in shifting the direction of N-transfer from the non-N_2-fixing plant to the N_2-fixing plant. Brophy *et al.* (1987) pointed out that N from grasses could be transferred to associated legumes, and Tomm *et al.* (1994) reported the occurrence of a bi-directional N-transfer between bromegrass (*Bromus riparius* Rhem.) and lucerne, although such transfer was not very significant under N-limited growing conditions. Hogh-Jensen and Schjoerring (2000) also demonstrated that approximately 8% of the above-ground N of the mixtures in white/red clovers (*Trifolium repens/T. pratense*) was derived from ryegrass. But the mycorrhization status of the tested plants was not observed in these three reports. Thus it is not clear that mycorrhizae were involved in these observations. Johansen and Jensen (1996) did report a very low N-transfer ($0.3 \pm 0.1\%$) from the non-legume bromegrass to the N_2-fixing legume lucerne and from barley to pea via AM hyphae. For such a small value, however, it was statistically difficult to show that N-transfer had genuinely occurred. More recently, Rogers *et al.* (2001) indicated that transfer of $^{15}NH_4$ occurred between white clover and ryegrass but it was independent of AM fungi because no AM hyphal links were observed by ^{14}C autoradiography. Moreover, intensive water movement under field conditions would unavoidably increase interspecific nutrient translocation between plants. As a result, the lack of convincing data underlines the fact that very carefully designed experiments are required in this intriguing area. Meanwhile, information of net N-transfer is also lacking since N-transfer from non-N_2-fixing plants to N_2-fixing plants has not been demonstrated. Furthermore, no study on either one-way or two-way N-transfer has been reported between Australian native trees.

So far, the following questions still remain to be answered:

- (1) Does N-transfer occur between N_2-fixing and non-N_2-fixing mycorrhizal plants at all? If so, how much N is transferred and how much is N-transfer enhanced by mycorrhizal hyphae?
- (2) How much N-transfer is from a non-N_2-fixing to an N_2-fixing mycorrhizal plant? That is to say, is N-transfer bidirectional? If yes, is transfer affected by mycorrhization and/or N_2-fixation?
- (3) Does N-transfer occur between any mycorrhizal plants, irrespective of their N_2-fixation characteristics?
- (4) Is mycorrhiza-mediated N-transfer of agricultural or ecological significance?
- (5) Can ^{15}N natural abundance be employed to detect N-transfer, similar to the ^{15}N enrichment technique?

These questions exist because almost all of the studies reported have been carried out under conditions in which the mycorrhizal plant root systems were not properly separated by an interposed solid plate, in order to avoid direct contact of roots but allowing a common hyphae link to be established through a fine nylon or stainless steel mesh. Secondly, almost all of them did not prevent the potential N-transfer between the plants through the water movement in the soil when the plants were routinely watered either in the glass house or in the field. Thirdly, almost all of them did not investigate the mycorrhiza-mediated N-transfer from the non-N_2-fixing plant to the N_2-fixing plant. That is, it is not known whether a net transfer of N from one plant to another occurs unidirectionally, or *vice versa* in which a two-way or bidirectional transfer might decrease the net N transfer. In addition, in studying the direct role of mycorrhizal hyphae in N-transfer between plants, it is important to keep in mind that mycorrhizae are four-way associations involving plants, fungi, N_2-fixing microbes and soils in N_2-fixing plants, or three-way associations in non-N_2-fixing plants. The lack of convincing data underlines the fact that very creative experimental manipulations and measurements are urgently required.

CHAPTER 2 MATERIALS AND METHODS

2.1 Plants, Growth Conditions and Harvest Procedures

2.1.1 *Casuarina cunninghamiana*

Casuarina is classified as one of the four genera in the family *Casuarinaceae*. The genus *Casuarina L.* has 17 species and almost all of them are Australian native plants (Midgley *et al.*, 1983; Pinyopusarerk *et al.*, 1996). Together with *Acacia* and *Prosopis* spp., *Casuarinaceae* are considered to be the most valuable multipurpose N_2-fixing perennials in the tropics and subtropics. Not only are they widely used as high quality fuel and timber wood, but also as effective soil improvers in nutrient-deficient soils. On the one hand, their root nodules (stem nodules even in some species) possess self-sufficient N-supplying capacity in symbiosis with *Frankia*. The range of 20–80% of biological N_2-fixation is found in different *Casuarina* species (Subbarao and Rodriguez-Barrueco, 1995). On the other hand, their root system can associate with either AM fungi of the genus *Glomus* or ECM fungi of the genus *Pisolithus* or both (Gauthier *et al.*, 1983; Gardner, 1986; Reddell *et al.*, 1986; Theodorou and Reddell, 1991; Subbarao and Rodriguez-Barrueco, 1995; Reddell *et al.*, 1997a; Osundina, 1998; Singh *et al.*, 1998; Mark *et al.*, 1999), which helps their host plants to access other nutrients and water through mycorrhization.

Casuarina cunninghamiana Miq., common name "river she-oak", is the largest species of the genus in Australia. From southern New South Wales to north Queensland and the Northern Territory, natural distributions of *Casuarina cunninghamiana* occur as pure stands in narrow riverine belts along fresh water streams, often at sites that are inundated periodically (Boland *et al.*, 1992). It also grows overlapping with several *Eucalyptus* species, including *E. camaldulensis* and *E. maculata*, mainly in the eastern areas of New South Wales and Queensland (Hills and Brown, 1978; Midgley *et al.*, 1983). The availability of N is very low in the soils of these native sites (Reddell, 1986). However, natural populations of *Casuarina cunninghamiana* invariably possess the capacity of fixing N_2 from the atmosphere within a range of 25–75%, especially by root nodules formed in symbiosis with

Frankia (McLuckie, 1923; Coyne, 1973; Subbarao and Rodriguez-Barrueco, 1995). In addition, *Casuarina cunninghamiana* is also capable of forming AMs with arbuscular fungi (Reddell *et al.*, 1986; Sempavalan *et al.*, 1995; 1996; Wheeler *et al.*, 2000) and ECMs with ectomycorrhizal fungi (Theodorou and Reddell, 1991), although a 30 % mycorrhization with *Pisolithus tinctorius* is relatively low.

Casuarina cunninghamiana can grow in warm sub-humid and semi-arid environments with 500 – 1,500 mm rainfall per annum within 20 – 1,000 m altitude and 12°S to 38°S latitude range. Because of its value for fuelwood, sand-binding and stream bank protection (Midgley *et al.*, 1983) and its capacity for tolerating frost up to –8°C (Turnbull, 1990), it has been widely introduced to the coastal areas of other countries such as China, Egypt, India, Israel, Senegal, and the United States. It can grow from a medium-sized to tall tree 20 – 35 m in height, 0.5 – 1.5 m in diameter and attain a wood density of 900 kg m^{-3} within a span of 10 years or so, but only requires minimum agronomic practices. In addition, its foliage can be used by stock due to its reasonably high nutritive value. These characteristics are very important for sustainable agriculture or agroforestry, especially in developing countries.

2.1.2 *Eucalyptus maculata*

Eucalypts have evolved predominantly on the Australian continent, where nutrient availability in most soils is inadequate to meet the nutritional demands of agricultural crops (Specht, 1996) and limits tree growth as well (Grove *et al.*, 1996; Florence, 1996). Indeed, the name *Eucalyptus* means "well covered" and the tree has become a symbol of the Australian biota (Florence, 1996; Williams and Brooker, 1997). The genus *Eucalyptus* belongs to the family *Myrtaceae* and includes about 700 species. Many species of *Eucalyptus* have been planted extensively for fuel, shelter, paper and pulp, and ornamental purposes around the world (Doughty, 2000), although most eucalypts are naturally grown in Australia (Eldridge *et al.*, 1993; Florence, 1996).

The conventional hypothesis (Beadle, 1957; Beadle, 1962a, b; Westoby, 1988; Noble, 1989) that growth and distribution of Australian native trees are significantly affected by the chemical content of soils and their parent materials has recently been

challenged (Adams, 1996). It is the biology of nutrient-cycling together with the availability of water, not the geochemical processes in the soils *per se*, that supplies most of the P, N and other nutrients for tree growth in native ecosystems (Adams, 1996). One important mechanism contributing to this is that *Casuarina* is one of the main N_2-fixing species in communities dominated by eucalypts. The range of N input from N_2-fixing plants is from 1 to 32 kg N ha^{-1} per year (Keith, 1997). Another important mechanism contribu ting to efficient nutrient uptake and water absorption by most eucalypts is the symbiosis between their fine roots and mycorrhizal fungi, either endomycorrhizal or ectomycorrhizal, or both (Chilvers *et al.*, 1987; Boudarga and Lapeyrie, 1990; Brundrett and Abbott, 1991; Bellei, 1992; Brundrett *et al.*, 1996; May and Simpson, 1997; Chen *et al.*, 2000).

Eucalyptus maculata, common name "spotted gum", is widely distributed in eastern Australia from near 38°S in Victoria to 25°S in Queensland, where there is 500 – 1,500 mm rainfall per annum. It can grow in coastal plains and hills from near sea level to 800 m altitude as a pure stand or a mixture with other eucalypts and other plants such as *Casuarina* (Florence, 1996). *Eucalyptus maculata* generally can attain between 20 – 30m height over 10 – 15 years with vigorous straight growth and good self pruning. On favourable sites the mature trees can reach 45 m and achieve a wood density of 800 kg m^{-3} within a span of 20 years or so. It has been widely cultivated and grows well in southern Africa, southern America and southeastern Asia. It is famous for its excellent timber quality, being used for preservative-treated transmission poles, heavy and general construction materials, flooring, sleepers, tool handles and so on, although it is not a very satisfactory pulp material (Hills and Brown, 1978).

2.1.3 *Glycine max* cv. Manark

The soybean belongs to the genus *Glycine* L. of the family *Leguminosae* and the major cultivated species is called *Glycine max* (L.) Merrill (Fageria *et al.*, 1995). Soybean was probably domesticated in northeastern China over 1,000 years ago and introduced into Europe in the early 1700s and into North America in the early 1800s (Whigham, 1983). Today, it is the most important crop legume crop in warm

temperate climates and its cultivation is increasing in the tropics and subtropics. It can be grown in a wide range of climates from 0 to 55° latitude, from sea level to 2,000 m elevation and on a wide range of soils with the optimum pH 6.0 – 6.5. At present, the top two soybean cultivation countries are the United States (28×10^6 hectares) and Brazil (12×10^6 hectares) (FAOSTAT, 2001). Soybean is often grown in rotation and/or intercropping with cereals such as corn, sorghum and millet in order to facilitate pest control, optimise available labor, and more importantly, to contribute N to cereals through its N_2-fixation (Peoples and Herridge, 1990, 1999; Fujita *et al.*, 1992; Stern, 1993; Chalk, 1996a, b; 1998).

Soybean starts to fix atmosphere N_2 approximately 4 weeks or can be earlier after germination if the proper *Rhizobium* bacteria are present in its roots. In most cases, the fixed N_2 accounts for 25–75% of the plant total N and is in the range of 60–108 kg N ha^{-1} $year^{-1}$ (Deibert *et al.*, 1979; Fujita *et al.*, 1992). Nitrogen fixation is promoted by early supply of lower level mineral NH_4^+ or NO_3^- to stimulate plant establishment and nodule development, but is inhibited by high levels of mineral N (Marschner, 1995). In addition, other essential nutrients especially P, Mo and Co should be supplied in appropriate amounts in order to obtain optimum N_2-fixation and seed yield. At harvest, 1 tonne of soybean grain removes approximately 59 kg N, 60 kg P, 19 kg K (Fageria *et al.*, 1995) and limited amounts of other nutrients as well. To maintain soil fertility, therefore, the nutrients (other than the same N) that were removed with the grain should also be returned in fertiliser and/or manure. Meanwhile, soybean has developed symbiosis with AM fungi to obtain nutrients from soils. The root mycorrhizal infection varies greatly with environmental conditions. In general, 20-70% root colonisation for soybean (Bethlenfalvay *et al.*, 1991; Hamel and Smith, 1991; Hamel *et al.*, 1991; Sieverding, 1991; Hamel and Smith, 1992; Martin *et al.*, 1995; Bethlenfalvay *et al.*, 1997, 1999; Khalil *et al.*, 1999; McGonigle *et al.*, 1999; Mujica *et al.*, 1999; Sanginga *et al.*, 1999; Ezawa *et al.*, 2000; Shrihari *et al.*, 2000; Auge *et al.*, 2001; Kelly *et al.*, 2001) has been observed.

The importance of soybean is due to the high protein and oil content of its grain. By dry weight, soybean seeds contain 40% protein, 21% oil, 26% carbohydrate and a

small amount of mineral nutrients (Johnson and Bernard, 1962) and thus provide a valuable food for both human and livestock consumption. The oil is used for human food, various pharmaceuticals and medicines, and making disinfectants, printing inks and soaps. The seeds can be manufactured into numerous edible products such as the fresh and dry *Tofu*, fermented food, soy beverage, flour, whole-bean confectionery, as well as textural vegetable protein used as simulated meat, fruit and nut products (Whigham,1983; Mounts *et al.*, 1987).

The world average production and yield of soybean were 136.3 million tonnes and 2.11 t ha^{-1}, respectively, in 1990–2001 (Table 2.1). However, its yield can be as high as 7.4 t ha^{-1} (Gabel, 1979). In general, soybean production is at least 3 times that of cotton and 4 times that of peanut, sunflower and oilseed rape. Due to an increased market demand, soybean production has been doubled since 1970 and consisted of 32% in 1965, 55% in 1980 and 89% in 1993/1994 of the whole world oilseed market (Smith and Huyser, 1987; Commodity Statistical Bulletin, 1998; Fageria *et al.*, 1995).

With an average annual yield of 1.86 t ha^{-1}, which was below the world's mean yield, the average annual area of soybean planted was 39,850 hectares and the average annual soybean production was 75,000 tonnes in Australia from 1990 to 2001 (Table 2.1). Of this, 50% was in southeast Queensland and 49% was in northeast New South Wales (Commodity Statistical Bulletin, 1998). Soybean annual production in Australia declined below the average in the period from 1991 to 1996 (Table 2.1), except in 1994, because alternative crops were planted and because of drought conditions (Colton, 1994). However, more recently (1998 to 2001) production has reached around 100,000 tonnes, mainly because of the continuously expanding demand by both the international and domestic markets.

2.1.4 *Sorghum bicolor* cv. New Nugget

Sorghum (*Sorghum bicolor* L. Moench) is a warm temperate and tropical cereal with the C_4 photosynthetic pathway and ranked fifth among cereals behind wheat, rice, corn and barley in worldwide area planted and production (FAOSTAT, 2001; Smith

and Frederiksen, 2000). Over 55% of the global sorghum production is in the semi-arid tropics. Of this, about 65% is from Asia and Africa, of which 34% is harvested in India (Sahrawat *et al.*, 1996; Smith and Frederiksen, 2000). There is evidence of sorghum in Assyria by 700 B. C. and in India and Europe by A. D. 1 (Eastin, 1983; Maiti, 1996). Cultivated sorghum originated later in Ethiopia and Sudan in northeast Africa and spread from there to West Africa (Doggett, 1970; Maiti, 1996). It was first introduced to America and Australia about 100 years ago and then spread throughout the world (Fageria *et al.*, 1995). The cultivation of sorghum is concentrated between latitudes 45°N and 40°S and occurs on a wide range of soils with pH 5.0 to 8.5 (Doggett, 1970; Purseglove, 1985). It is a drought-resistant, moderately salinity tolerant and low fertiliser requiring crop, although its cultivars differ in their reactions to these conditions (Shih *et al.*, 1981; see Fageria *et al.*, 1995; Smith and Frederiksen, 2000), and it is often grown in areas that are too dry to grow corn.

Table 2.1. Soybean production between 1990–2001 in Australia and the World (FAOSTAT, 2001).

Year	Planted Area (ha)		Yield (t ha^{-1})		Production (t)	
	Australia	World (10^6)	Australia	World	Australia (10^5)	World (10^6)
2001	55,000	75.54	1.91	2.34	1.05	176.64
2000	52,000	73.44	2.00	2.21	1.04	161.99
1999	53,000	71.85	1.98	2.19	1.05	157.31
1998	48,000	70.79	2.27	2.26	1.09	159.96
1997	39,000	66.95	1.90	2.16	0.74	144.41
1996	23,803	61.08	1.87	2.13	0.45	130.21
1995	17,787	62.50	1.53	2.03	0.27	126.91
1994	40,633	62.48	2.00	2.18	0.81	136.46
1993	30,239	59.51	1.62	1.94	0.49	115.23
1992	29,611	56.16	2.11	2.04	0.63	114.45
1991	39,880	54.96	1.56	1.88	0.62	103.31
1990	49,251	57.18	1.57	1.90	0.77	108.45
Mean	***39,850***	***63.37***	***1.86***	***2.11***	***0.75***	***136.28***

The world average production and yield of sorghum were 60.5 million tonnes and 1.39 t ha^{-1} in 1990–2001 (Table 2.2); yields range from as low as 0.66 t ha^{-1} in parts of Africa to as high as 4.00 t ha^{-1} in Latin America (Peacock and Wilson, 1984), but the potential yield is 14.25 t ha^{-1} (Fischer and Wilson, 1975). Sorghum has been cultivated for food since ancient times in Africa and India and today is still the basic

cereal food in parts of Africa and Asia, while in the United States, Europe and Australia it serves mainly as feed for poultry and livestock. Sorghum stems and foliage are often used as animal fodder and in some areas the stems are used as building material and fuel. It is also widely used for brewing beer, wine, and industrial ethanol as well. Some sorghum species have sweet, juicy stems that contain up to 10% sucrose and are chewed or used to make syrup. At harvest one tonne of sorghum grain removes about 18 kg N, 3 kg P, 4 kg K (Fageria *et al.*, 1995) and a certain amount of other nutrients as well. To maintain soil fertility and improve sorghum yield, both soil and plant analysis can be used for diagnosis and correction of nutrient deficiencies and toxicities. Sorghum can also develop an endomycorrhizal symbosis with AM fungi to capture nutrients from soils. The root mycorrhizal infection of sorghum varies greatly with environmental conditions. In general, 20-60% of root colonisation (Graham *et al.*, 1982; Ocampo, 1986; Raju *et al.*, 1990; Hawkins and George, 1997; Brundrett *et al.*, 1999; Caris *et al.*, 1999; Godeas *et al.*, 1999; Abdel-Fattah and Mohamedin, 2000; Bagayoko *et al.*, 2000a, b; Fonseca *et al.*, 2001) has been observed.

The average annual area planted to sorghum in Australia was 549,301 hectares and the average annual yield was 2.34 t ha^{-1} from 1990 to 2001 (Table 2.2). Although both the total planting area (from 377,801 in 1991 to 769,800 hectares in 1996) and mean yield (from 1.28 in 1993 to 3.33 t ha^{-1} in 2000) fluctuated widely between 1990 and 2000 (Table 2.2), it remains a major crop in many farming systems (Borrell and Henzell, 2001). Also, there is an increasing trend for its demand in both international and domestic markets. Of the total sorghum production in Australia, 50% to two thirds were produced in southeast Queensland, the remainder largely in northeast New South Wales (Commodity Statistical Bulletin, 1998). In Australia, most of the sorghum is used as feed-stuff in the domestic dairy industry, instead of a major food for the people, as is the case in many semi-arid tropical regions. Some sorghum is exported overseas or used for a variety of industrial products.

2.1.5 *Experimental design*

The specific individual experimental designs are outlined in Chapters 3 and 4. The central hypothesis was that mycorrhiza-mediated bidirectional N-transfer and N

movement is determined by dynamic four-way interactions among plant roots, mycorrhizal fungi, N_2-fixing bacteria, and N resource availability and requirements. Four ^{15}N labeling experiments, 98.0 atom $\%^{15}N$, either as $^{15}NH_4^+$, $^{15}NO_3^-$, $^{15}NH_4{}^{14}NO_3$ or $^{14}NH_4{}^{15}NO_3$, was fed for 4 weeks to only one species, designated as the 'N-donor', and N-starvation applied to the adjacent species, as the 'N-receiver',

Table 2.2. Sorghum production between 1990–2001 in Australia and the World (FAOSTAT, 2001).

Year	Planted Area (ha)		Yield (t ha^{-1})		Production (t $\times 10^6$)	
	Australia	World $\times 10^6$	Australia	World	Australia	World
2001	596,000	42.64	2.39	1.36	1.42	58.15
2000	648,000	42.07	3.33	1.39	2.16	58.50
1999	587,000	42.10	3.22	1.43	1.89	60.33
1998	507,000	43.16	2.13	1.43	1.08	61.18
1997	544,000	43.87	2.62	1.39	1.42	62.63
1996	769,928	46.99	2.07	1.52	1.59	71.64
1995	686,246	42.74	1.86	1.28	1.27	54.57
1994	499,461	44.41	2.17	1.36	1.08	60.37
1993	427,000	41.32	1.28	1.38	0.55	56.09
1992	569,137	46.28	2.54	1.52	1.45	70.57
1991	377,801	42.89	1.99	1.30	0.75	55.67
1990	380,034	41.59	2.49	1.36	0.95	56.71
Mean	***549,301***	***43.34***	***2.34***	***1.39***	***1.30***	***60.53***

before harvesting. This approach artificially established a ^{15}N concentration gradient between the 'N-donor' and the 'N-receiver', and enabled detection of the ^{15}N isotope received by one species from the other. The non-mycorrhizal control pairs acted as an indicator for ^{15}N movement through soil pathways. The negligibly low ^{15}N in the control receivers indicated that the transfer between mycorrhizal pairs was through the direct hyphal pathway, and not the soil pathway, irrespective of whether $^{15}NH_4^+$ or $^{15}NO_3^-$ was applied (NO_3^- is more chemically mobile than NH_4^+). The non-mycorrhizal non-N_2-fixing/sole mycorrhizal (non-nodulated) N_2-fixing plant pairs validated both hyphal interconnection and mycorrhiza-mediated ^{15}N-transfer from one species to another, as seen in the sole mycorrhizal/mycorrhizal pairs without N_2-fixation intervention. The dual nodulated mycorrhizal N_2-fixing/mycorrhizal non-N_2-fixing plant pairs were used to examine further if such N-transfer was also influenced by N_2-fixation. The non-mycorrhizal nodulated N_2-fixing/mycorrhizal non-N_2-fixing plant pairs validated the participation of both N_2-fixation and the hyphal link. Two-

way (bidirectional) or net transfer was the sum or the difference, respectively, between N transferred to *Casuarina* (or soybean) and to *Eucalyptus* or (*Sorghum*).

For $\delta^{15}N$ abundance experiments, the external ^{14}N source was supplied continuously to both the 'N-donor' and the 'N-receiver' for a certain period of time, and then only the 'N-receiver' was deprived of N at 4 weeks before harvesting. A similar approach, as outlined above in the ^{15}N experiments, is generally adopted to trace N-transfer between plants from the change of $\delta^{15}N$ values.

2.1.6 *Experimental growth unit*

Plants were grown in 5.0 L (300 × 12 × 150 cm) plastic boxes consisting of two equal halves (Figure 2.1). These two compartments restrict the root growth of donor and receiver to their own halves. The compartment was sealed by two perspex plates with paraffin wax to produce a third 10 mm narrow air gap bridge compartment for mycorrhizal hyphae contact. Each plate contained 312 × 6 mm diameter holes and was covered on each side with 37 μm nylon mesh (Nytal® Swiss Screen).

2.1.7 *Plant growth conditions*

Casuarina and *Eucalyptus*, and soybean and *Sorghum*, were paired as either N-donor or N-receiver plants. Three donor seedlings were grown in one compartment of the growth unit and 3 receivers in the other, in a Controlled Environment Chamber (28/23°C, day/night) located at a University of Queensland (UQ) glasshouse. The plants were watered weekly with 5–10 mL N- or N-free nutrient solution described by Brundrett *et al.* (1996) and daily with 5 – 20 mL deionised water depending on plant vigour. The surface of the growth medium in each compartment was covered by a suitable layer of high density polythene resin beads (75% ethanol soaked and sterilised water rinsed) to reduce water loss. The bases of the growth units were wrapped in aluminium foil to protect the roots from sunlight. The growth boxes were randomly repositioned twice weekly to keep light exposure uniform.

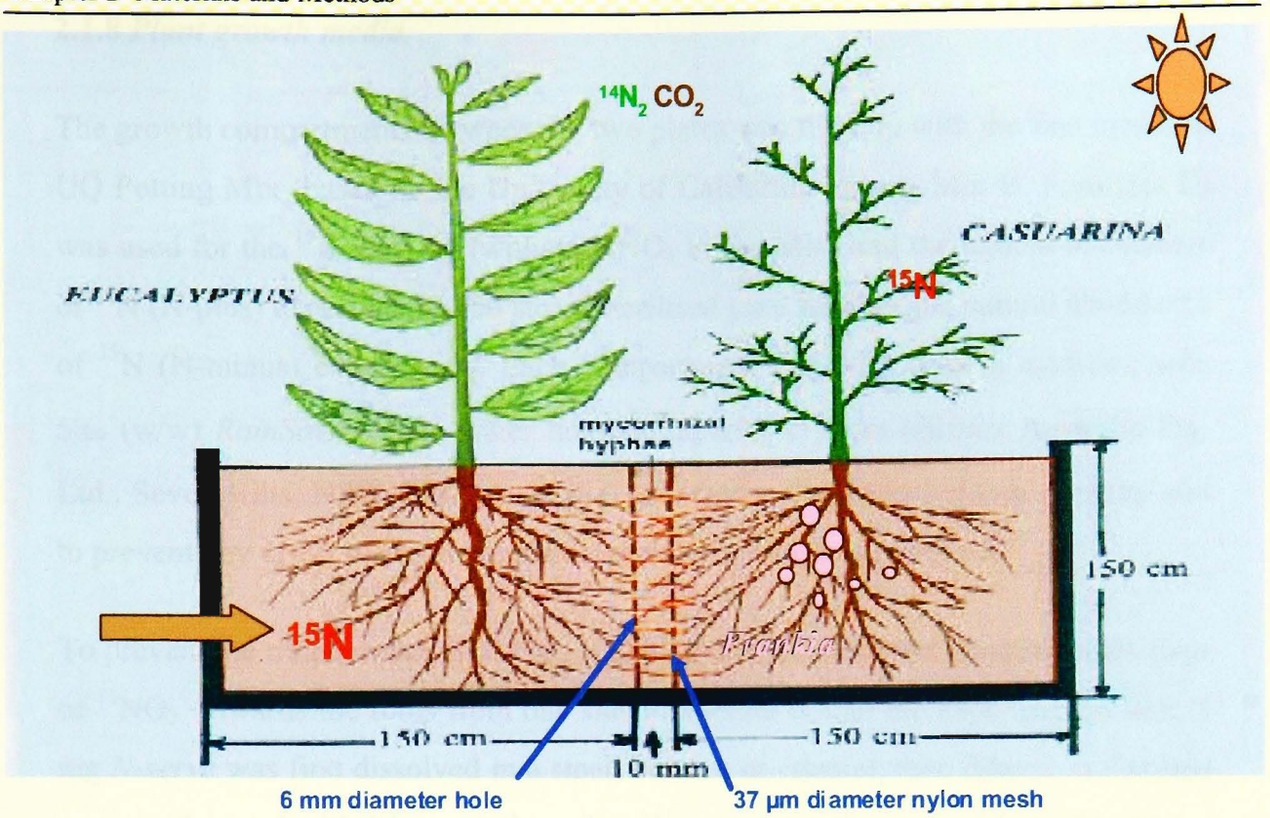

Figure 2.1. Longitudinal section of a growth container with two perspex plates covered by 37µm nylon mesh separating the root systems of *Casuarina* and *Eucalyptus* growing in individual root compartments.

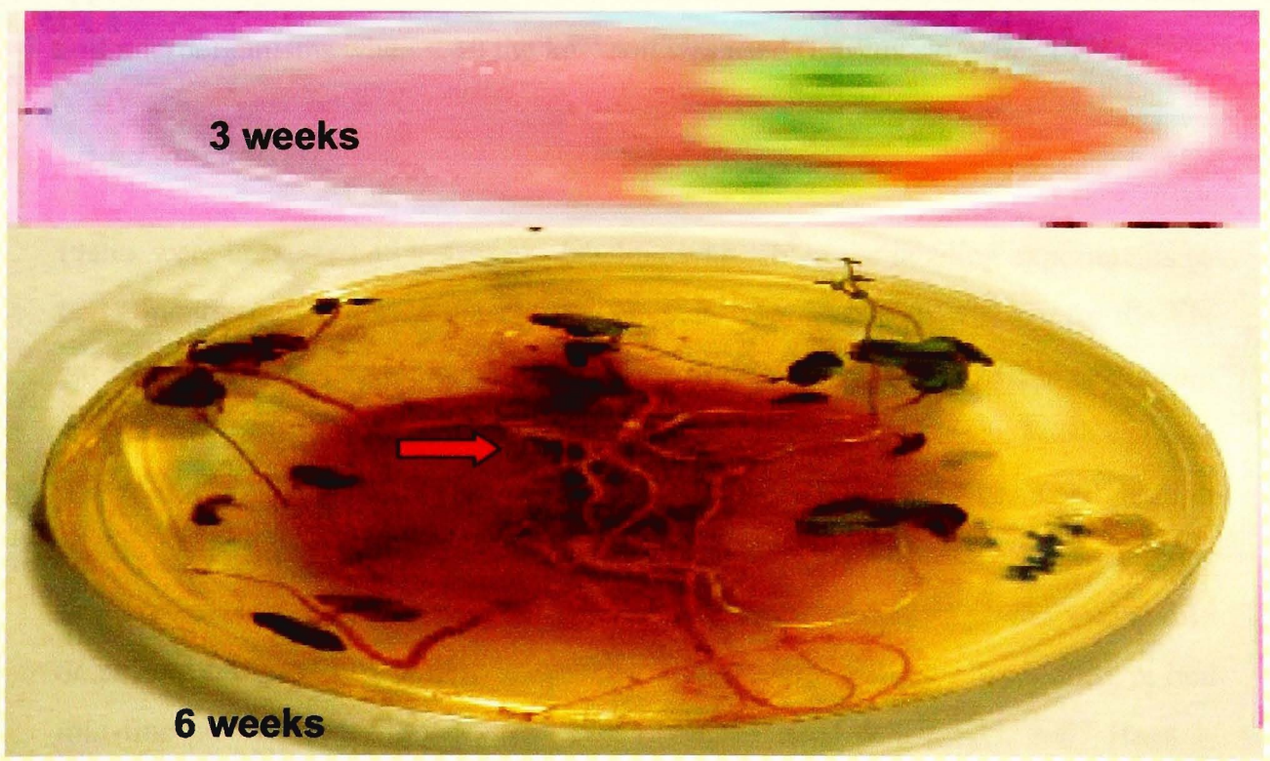

Figure 2.2. Aseptic subculture of ectomycorrhizal fungus *Pisolithus tinctorius* (top) and ectomycorrhizal seedling generation on agar media in Petri dishes (bottom) (red arrow: mycorrhizal tips).

2.1.8 *Plant growth media*

The growth compartments between the two plates was filled in with the fine medium. UQ Potting Mix (based on the University of California Potting Mix B, Fertilizer II) was used for the ^{15}N labeling (without KNO_3 in the Mix) and the natural abundance of ^{15}N (N-plus) experiments, and steam sterilised pure sand for the natural abundance of ^{15}N (N-minus) experiments. Each compartment had 3 kg growth medium, with 5‰ (w/w) *RainSaver*® high-water holding capacity crystals (Hortex Australia Pty. Ltd., Seven Hills, NSW 2147, Australia) to increase the water holding capacity and to prevent any direct water movement between the two compartments.

To prevent the transformation of NH_4^+ to NO_3^- and minimise the resulting mass flow of $^{15}NO_3^-$ towards the roots from one side to another within the experimental unit, 5 mg *N-serve* was first dissolved in a small volume of ethanol, then diluted in distilled water and mixed with 1 kg growth media. *N-serve*, a nitrification inhibitor [2-chloro-6-(trichloromethyl) pyridine; Sigma-Aldrich Pty Ltd., Castle Hills, NSW 2154], retards the first step in the nitrification of NH_4^+ to hydroxylamine by *Nitrosomonas* spp. (see Slangen and Kerkhoff, 1984). *N-serve* has not been found to affect other soil microorganisms when < 10 mg kg^{-1} soil was used (Laskowski *et al.*, 1975).

2.1.9 *Plant harvest*

Plants were harvested after 4-weeks labeling with ^{15}N in the labeling experiments (6 or 12-months cultivation in glasshouse) or at the appropriate growth age for the ^{15}N natural abundance experiments (experiments within 6 or 12-months for *Casuarina* and *Eucalyptus*, or 3-months for soybean and *Sorghum* in glasshouse). Fresh samples were collected before harvesting and preserved in 50% ethanol and/or in methanol in tightly sealed screw top vials for root mycorrhizal infection and/or for other analyses. Dry materials of shoots, roots and nodules were oven-dried at 59°C and weighed to determine biomass production. Randomly selected dry materials were then milled into fine powder in a vibratory ball mill (Retsch GmbH & Co. KG, 5657 Haan 1, Germany) and kept in tightly sealed screw top vials for total N, ^{15}N and $\delta^{15}N$ analyses.

2.1.10 *Xylem fluid collection*

Xylem fluid in both stem and root was collected in the morning around 10:30am using a hand vacuum pump according to Pate *et al.* (1994). Plant leaves were removed, stem was cut and the bark including phloem was scraped from the bottom of the stem or the top of the root. Then, the bottom or the top end was shaped to fit 100 – 1,000 μL pipette tips. A seal adhesive [Bostik Blue•Tack, Bostik (Australia) Pty. Ltd., Victoria 3074, Australia] was wrapped around the stump before revolving it into the pipette tip. The pipette tip was then inserted into a rubber bung sealing a 25 mL plastic vial which contained a micro-centrifuge tube with a volume of 2 mL. The xylem fluid was extracted by applying the vacuum of up to −80 kPa and simultaneously cutting 0.5cm pieces off the stem or the root. The fluid samples were kept on ice and transferred into a freezer for storage.

2.2 Growing N_2-fixing and/or Ectomycorrhizal Seedlings

2.2.1 *Plants*

Seeds of *Casuarina cunninghamiana* and *Eucalyptus maculata* were purchased from the Forestry Seed Centre, Department of Primary Industry, Beerwah, Queensland 4519. Seeds were surface sterilised with 70% ethanol for 30 seconds and in 15% H_2O_2 for 5 minutes, rinsed 5 times with sterilised water, and then germinated on modified 1.0% (w/w) agar [ammonia media with 1.0 % (w/v) agar but without biotin and vitamin B_{12}] in a Petri dish according to Ahmad and Hellebust (1991).

2.2.2 *Aseptic culture of the ectomycorrhizal fungus Pisolithus tinctorius*

The ectomycorrhizal fungus *Pisolithus tinctorius* (Pers.) Coker and Couch was used to inoculate the tree seedlings. It was kindly supplied by Drs P. McGee and W. Allaway (School of Biological Sciences, The University of Sydney, New South Wales 2006 Australia, originally isolated by S. M. Chambers from a fruit body collected by J. G. Cairney in 1988 from coastal heath at North Head, Sydney). The methods for the fungus subculture in a Petri dish and the isolate maintenance of a 3

months periodic culture transfer regime were accorded to Brundrett *et al.* (1996). The fungal mycelia began growing visibly on the agar media after 3 weeks incubation at room temperature without light exposure (Figure 2.2).

2.2.3 *Generation of aseptic ectomycorrhizal seedlings*

The method for formation of an ectomycorrhizal association between *Casuarina* or *Eucalyptus* and *Pisolithus tinctorius* was adapted from Brundrett *et al.* (1996) and Theodoru and Reddell (1991). The fungus was subcultured either in sealed axenic 90 mm × 10 mm or in 140 mm × 20 mm Petri dishes (Nclon™ Surface, Nalge Nunc International, Denmark) on the nutrient agar. Five or nine square agar mats (4 × 4 mm) with or without fungal mycelia were placed onto fresh nutrient agar in the Petri dishes for mycorrhizal formation. Fungal growth was apparent after 3 weeks (Figure 2.2). Six to 10 axenically germinated *Casuarina* or *Eucalyptus* seedlings were placed onto the fungal cultures, and incubated in a slanted upright position in a fungal culture room (25°C and a light level of 200 μE m^{-2} s^{-1}). Mycorrhiza formation was visible after approximately 6 weeks incubation (Figure 2.2). The Petri dishes were then gradually opened and seedlings underwent acclimatisation for 1 week in non-totally-sealed dishes. The acclimatised, relatively uniform seedlings were out-planted into cylindrical polythene zip-lock bags (90 cm × 150 cm) for 2 months, and were then transplanted into the growth unit and grown in the glasshouse until harvest (see 6-months-old plants in Figure 2.3).

2.2.4 *Frankia inocula and collection of Frankia nodules*

The *Casuarina cunninghamiana* seedlings were originally inoculated with pure *Frankia* strains UGL020604 and UGL020605 (kindly supplied by Dr. C. T. Wheeler, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow GI2 8QQ UK) and grown in a glasshouse on the UQ campus. These *Frankia* strains are highly effective on *C. cunninghamiana* (Sempavalan *et al.*, 1996). Nodulation of roots began after 6 weeks growth and was checked routinely. To make *Frankia* inocula, fresh and healthy *Frankia* nodules around 5 mm in diameter were directly collected

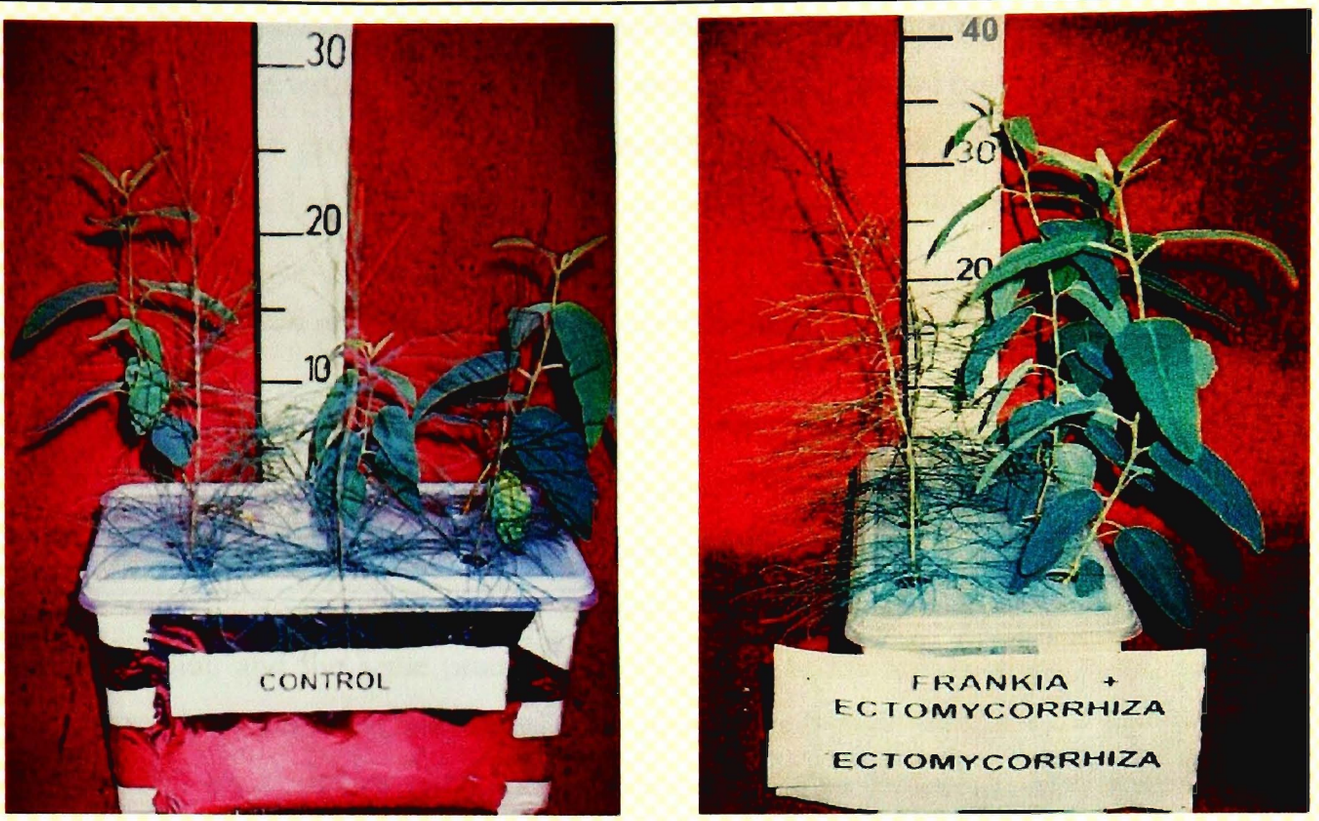

Figure 2.3. 6-months-old mycorrhizal and non-mycorrhizal *Casuarina* and *Eucalyptus* growing in the glasshouse. The mycorrhizal plants were much taller and stronger than their controls.

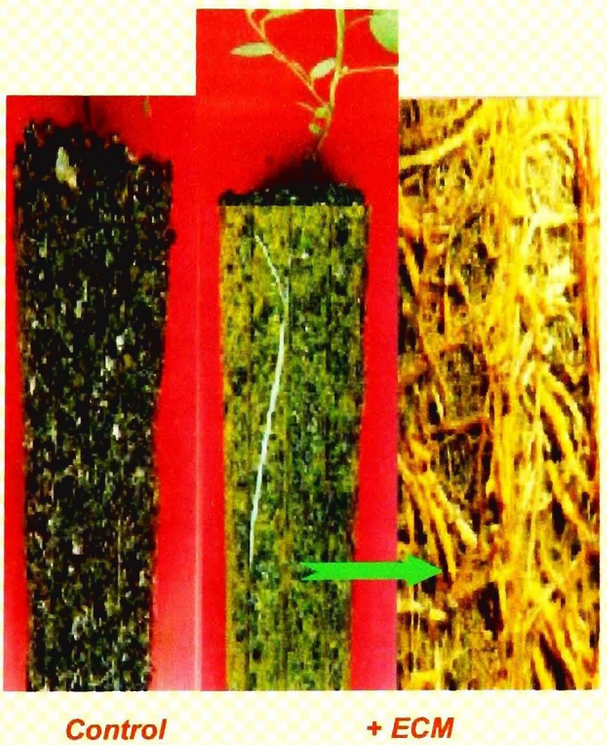

Figure 2.4. ECM in 12-months-old *Eucalyptus* roots. No infection in control roots, whereas enormous yellow tips in infected roots. Green arrow: magnified tips.

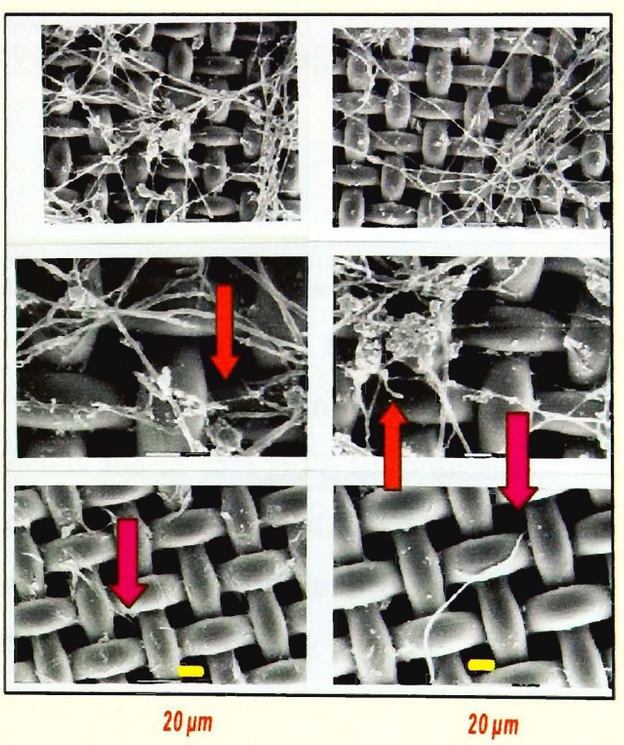

Figure 2.5. ECM in 12-months-old *Casuarina*, as seen under an Environmental Scanning Electron Microscope. Red arrows: mycorrhizal tips; Pink arrows: hyphae across nylon mesh.

from these nodulated *Casuarina cunninghamiana* roots. The nodules were surface sterilised twice with 70% ethanol for 30 seconds, rinsed several times with sterilised water, and then ground to a paste in a mortar and pestle with sterilised water. The paste was mixed and filtered through gauze, and then diluted with sterilised water.

2.2.5 *Generation of N_2-fixing or ectomycorrhizal N_2-fixing seedlings*

The acclimatised non-mycorrhizal and mycorrhizal *Casuarina* seedlings were first dipped in fresh *Frankia* suspension for 20 minutes and 5 mL of this suspension was also added to the root base during transplantation, and again 1 and 3 weeks after transplanting to ensure successful inoculation. The same amount of boiled nodule suspension and the same procedure were applied to the non-N_2-fixing seedlings. These plants were then out-planted into cylindrical polythene zip-lock bags (90 cm × 150 cm) for 2 months growth to develop root nodules. Relatively uniform, well-nodulated and non-nodulated seedlings were then selected for transplantation into the growth unit and grown in the glasshouse until harvest.

2.3 Growing N_2-fixing and/or Endomycorrhizal Seedlings

2.3.1 *Plants*

Seeds of soybean (*Glycine max* L. cv. Manark) and sorghum (*Sorghum bicolor* L. cv. New Nugget) were kindly provided by Dr. S. Fukai, School of Land and Food Sciences, The University of Queensland, Brisbane, QLD 4072 Australia. Seeds were surface sterilised with 70% ethanol for 30 seconds and in 15% H_2O_2 for 5 minutes, rinsed 5 times with sterilised water, and then germinated on modified 1.0% agar [ammonia media with 1.0 % (w/v) agar but without biotin and vitamin B_{12}] in Petri dishes according to Ahmad and Hellebust (1991).

2.3.2 *Pot culture of the endomycorrhizal fungus Glomus mosseae*

A pure strain of arbuscular mycorrhizal fungus, *Glomus mosseae* (Nicol. and Gerd.) (kindly supplied by Dr. V. Galea, School of Agriculture and Horticulture, The

University of Queensland, Lawes, Queensland 4343 Australia) was propagated on sorghum grown in autoclaved sands in pots in a University of Queensland glasshouse for 10 weeks according to the method of Brundrett *et al.* (1996).

2.3.3 *Generation of endomycorrhizal seedlings*

100 g of *Glomus mosseae* pot culture or an autoclaved equivalent materials, consisting of sand, spores, hyphal fragments and heavily colonised sorghum fine roots, was placed at middle depth in each compartment of the growth unit containing 3 kg steam sterilised pure sand. Three relatively uniform soybean or sorghum seedlings were transplanted in one of the two compartments in the growth unit.

2.3.4 *Rhizobium inocula and inoculation of soybean seedlings*

A commercial legume inoculant for soybean called *Nitri-life* (multi-strains of rhizobia) was purchased from Inoculant Services, Bethanga, Victoria 3691 Australia. To make rhizobium inocula, 50 g of *Nitri-life* and 1 % sucrose (w/v) were dissolved in 500 mL of sterilised water on a magnetic stirrer for 1 hour on ice in a laminar flow hood. The inocula were directly applied to germinated soybean roots for 10 minutes and again to the root base after the plants had grown in the growth unit for 1 week. Boiled inocula were applied to the control plants.

2.4 Determination of Mycorrhizal Associations

2.4.1 *Light microscopy*

Figure 2.4 shows the mycorrhization status of *Eucalyptus*. The percentage of total mycorrhizal infection (% root length colonised) of both ectomycorrhizal and arbuscular mycorrhizal colonisations was determined by the grid line intersection method (Giovannetti and Mosse, 1990; Brundrett *et al.*, 1996). AM association was also examined by Dr. Victor Galea, School of Agriculture and Horticulture, The University of Queensland, Lawes, Queensland 4343 Australia.

2.4.2 *Environmental scanning electron microscopy*

Living plants were taken directly to the Centre for Microscopy and Microanalysis, The University of Queensland, for non-destructive observation. Fresh fine roots were examined with an ElectroScan E3 Environmental Scanning Electron Microscope (Electro-Scan Corp., Wilmington, Massachusetts, USA) for EM or AM associations and CMN linkages between pairs of casuarinas, eucalypts, casuarina/eucalyptus with the ECM fungus *P. tinctorius*, or soybean/sorghum with the AM fungus *G. mosseae*. Figure 2.5 shows the mycorrhization of *Casuarina* roots. The fungus mycelia ramified over the nylon mesh and the hyphae crossed the mesh holes to establish common mycorrhizal linkages between plants.

2.5 Nitrogen Assays

Two mg samples were weighed into a tin capsule for % N and ^{15}N atom % excess determination. For ^{15}N natural abundance ($\delta^{15}N$ ‰) value, samples (≤15 mg) containing approximately 130 μg of N were then analysed by an Automated $^{15}N/^{13}C$ Analyser-Mass Spectrometer (ANCA – MS; Europa Scientific, Crewe, UK). Based on multiple analysis of the laboratory standard at UQ Botany with *Eucalyptus crebra* leaves, the precision of this instrument for $\delta^{15}N$ was 0.12‰ standard deviation. Some nitrogen assays were performed with the same type of ANCA – MS at the Waikato Isotope Unit, The University of Waikato, Hamilton, New Zealand.

2.6 Amino Acid Assays

Amino acids in the stem and root xylem fluid [fresh leaf, root and nodule samples were initially extracted in methanol (w/v, 1:10) and kept in the freezer at – 20°C] were determined by an HPLC – based amino acid analyser (System 6300, Beckman Instruments, Palo Alto, California, USA) with a post – column ninhydrin derivatisation method. The detection limit was > 1 nmol g^{-1} FW.

2.7 Chlorophyll Assays

Fresh leaf samples were extracted in methanol (w/v, 1:10) and kept in the freezer at – 20°C. Chlorophyll *a*, *b* and (*a* + *b*) concentrations were determined using a Model

DU 640 Scanning Spectrophotometer (Beckman Instruments, Palo Alto, California, USA). Correction factors and equations for calculating chlorophyll content followed the methods of Porra *et al.* (1989).

2.8 N_2-Fixation and N-transfer Calculations

2.8.1 *Nitrogen fixation (please also see section 1.2 of Chapter 1)*

Nitrogen fixation was calculated by

1. the specific nodule activity: mg N_2 fixed g^{-1} DW nodule (1)

2. percentage of biological nitrogen fixation ($\%N_{BNF}$):

   a. for enriched ^{15}N samples by:

$$N_{fixed}=(1-atom\%^{15}Nexcess_{fixing\ plant}/atom\%^{15}Nexcess_{non\text{-}fixing\ plant})\times N_{fixing\ plant} \quad (2)$$

   and

   b. for natural abundance samples by:

$$N_{fixed}=(\delta^{15}N_{non\text{-}fixing\ plant}-\delta^{15}N_{fixing\ plant})/(\delta^{15}N_{non\text{-}fixing\ plant}-B) \quad (3)$$

   where the value "B" refers to the $\delta^{15}N$ value of the effectively nodulated N_2-fixing plant grown in media totally lacking external N.

2.8.2 *Calculation of N-transfer between plants (also see section 1.3 of Chapter 1)*

A. For enriched ^{15}N samples, percentage of N-transfer (% $N_{transfer}$), the amount of N (mg $plant^{-1}$) transferred from the donor ($N_{transfer}$) and the % of N in the receiver derived from transfer (% NDFT) were calculated by the following equations (Johansen and Jensen, 1996):

a. % $N_{transfer}$:

$$^{15}N_{content\ plant} = atom\%^{15}N\ excess_{plant} \times total\ N_{plant} / atom\%^{15}N\ excess_{labeled\ N} \quad (1)$$

$$\%\ N_{transfer}=^{15}Ncontent_{receiver}\times 100/(^{15}Ncontent_{receiver}+^{15}Ncontent_{donor}) \quad (2)$$

where

$$^{15}N_{content\ plant} = atom\%^{15}N\ excess_{plant} \times total\ N_{plant} / atom\%^{15}N\ excess_{labeled\ N} \quad (3)$$

b. The amount of N (mg plant^{-1}) transferred from the donor ($N_{transfer}$):

$$N_{transfer} = \%\ N_{transfer} \times total\ N_{donor} / (100 - \%N_{transfer}) \quad (4)$$

c. The % of N in the receiver derived from transfer (% NDFT):

$$\%\ NDFT = N_{transfer} \times 100 / total\ N_{receiver} \quad (5)$$

The following equation was also used for calculation % NDFT (Tomm *et al.*, 1994):

$$\%\ NDFT_{donor} = (atom\%^{15}N\ excess_{receiver} / atom\%^{15}N\ excess_{donor}) \times 100 \quad (6)$$

It was observed that the two equations (5) and (6) were equally matched if the 0.3663% ^{15}N of atmospheric N_2 was subtracted from the measured atom%^{15}N excess. This validates the results from equations (2) and (4).

For natural abundance samples, all the equations mentioned above were used except for the calculation of % N-transfer. It is possible to use $\delta^{15}N$ values to determine how much of the N accumulated in the root and shoot of the receiver plants is derived from the donor plants, if isotopic changes were sufficiently large during plant establishment and growth (see section 1.3.3 of Chapter 1). For the specific plant pairs (see Chapters 3 and 4), the % N-transfer were calculated as follows:

a. between the pure stand of mycorrhizal *Eucalyptus* pairs (either from *EucalyptusA* to *EucalyptusB* or from *EucalyptusB* to *EucalyptusA*):

$$\%\ N_{transfer} = (\delta^{15}Nreceiver_{control} - \delta^{15}Nreceiver_{mycorrhizal\ treatment}) / \delta^{15}Nreceiver_{control} \times 100 \quad (7)$$

Equation 7 was also used for the N-transfer calculation either from soybean to sorghum or from sorghum to soybean, as the $\delta^{15}N$ values in both species were positive in the experiments.

b. between the pure stand of mycorrhizal *Casuarina* pairs:

1. adding all the measured $\delta^{15}N$ values of samples with the $\delta^{15}N$ value of the reference plant (cultivated with N-free solution); then

2. using all the new calculated values in the following equations (either from *CasuarinaA* to *CasuarinaB* or from *CasuarinaX* to *CasuarinaY*):

$$\% N_{transfer} = (\delta^{15}N receiver_{mycorrhizal\ treatment} - \delta^{15}N receiver_{control}) / \delta^{15}N receiver_{mycorrhizal\ treatment} \times 100 \quad (8)$$

c. Between the paired stand of mycorrhizal *Casuarina* and *Eucalyptus*:

c1: from the *Casuarina* to *Eucalyptus*:

$$\% N_{transfer} = (\delta^{15}N receiver_{control} - \delta^{15}N receiver_{mycorrhizal\ treatment}) / \delta^{15}N receiver_{control} \times 100 \quad (9)$$

c2: from the *Eucalyptus* to *Casuarina*:

1. adding all the measured $\delta^{15}N$ values of samples with the $\delta^{15}N$ value of the reference plant (cultivated with N-free solution); then

2. using all the new calculated values in the following equations:

$$\% N_{transfer} = (\delta^{15}N receiver_{mycorrhizal\ treatment} - \delta^{15}N receiver_{control}) / \delta^{15}N receiver_{mycorrhizal\ treatment} \times 100 \quad (10)$$

2.9 Statistical Analyses

Three seedlings per species were used in every experiment and each individual experiment was replicated 3 times to obtain nine samples. Measurements, expressed as means and standard errors, were calculated for replicate samples, except for the calculation of bidirectional and net N transfer, when the average of two pairs for 18 total samples was taken. Data of both mycorrhizal colonisation and N-transfer were first transformed by The Arc Sine Transformation to stabilise the variance, and then separately analysed by ANOVA procedures for N-donor and N-receiver (Sokal and Rohlf, 1995). Differences of treatment means were considered significant at $P \leq 0.05$ or 0.01 and compared by the T-method (Tukey's Honestly Significant Difference Method) in order to increase the confidence level.

CHAPTER 3 NITROGEN TRANSFER BETWEEN *CASUARINA CUNNINGHAMIANA* AND *EUCALYPTUS MACULATA* VIA COMMON ECTOMYCORRHIZAL NETWORKS: ^{15}N LABELING STUDIES

3.1 Introduction

Inorganic N in soil is available to plants either as NO_3^- or NH_4^+ (Haynes and Goh, 1978; Lee and Stewart, 1978; Clarkson, 1985; Bloom, 1988; Marschner, 1995; Forde and Clarkson, 1999; Hawkins *et al.*, 2000). In almost all agricultural soils, NO_3^- is the dominant form of N available to plants while NH_4^+ is not readily available due to its rapid nitrification. NO_3^- is reduced to NH_4^+ either in the root, or transported via the xylem to the shoot where it is reduced (Simpson, 1986; Campbell, 1996). NH_4^+, on the other hand, is generally metabolised in the root (Oaks and Hirel, 1985; Oaks 1992). However, for higher plants, NH_4^+ is the final inorganic N form required for incorporation into amino acids and subsequent conversion into other organic N-compounds (Pate and Layzell, 1990). The presence of NH_4^+ often depresses NO_3^- uptake when the two ions occur simultaneously (Marschner *et al.*, 1991; Paul and Clark, 1996; Kreuzwieser *et al.*, 1997; Brady and Weil, 2002). Therefore, depending on the availability of NH_4^+ or NO_3^- in the soil and/or in the plant body, and the nature of plants themselves, plant species differ in their preferred forms of N absorbed. Most agricultural crops grow better on NO_3^- (Malhi *et al.*, 1988) while most forest conifers grow well on NH_4^+ (Kronzucker *et al.*, 1997). Some species grow best when cultivated on a mixture of NH_4^+ and NO_3^- (Hageman, 1984), while others, especially those confined to nutrient-impoverished soils, can use either NH_4^+ or NO_3^- (Vessey *et al.*, 1990; Atkin, 1996).

The stable isotope ^{15}N can be used as a tracer to investigate N-transfer between plants (Fujita *et al.*, 1992; Stern, 1993; Chalk, 1996a, b; 1998). Several techniques have been developed since the first use of ^{15}N labeling in soil-plant studies by Norman and Krampitz in 1945. Examples are the $^{15}N_2$ labeling method, foliar labeling with ^{15}N (spray or injection), the split-root technique, the transplanting technique, the N-

difference method, and the ^{15}N-dilution method. They have all been used in various studies (Chalk, 1996a, b). Among the techniques, the most satisfactory method is $^{15}N_2$ labeling, since ^{15}N detected in the plant will be solely derived from N_2-fixation (McNeill and Wood, 1990). However, this method requires enclosure of the whole plant or the root system. The ^{15}N-dilution method is now the most widely used technique for estimating N-transfer in intercrops and also in mixed systems since it was introduced by Vallis *et al.* in 1967 (Peoples and Herridge, 1990; Chalk, 1996a, b; 1998). This method depends on the use of paired plots containing the non-N_2-fixing plant in a pure system and in a mixed system with the N_2-fixing plant. It assumes that both the non-N_2-fixing plants and the N_2-fixing ones absorb the same relative amounts of N from the enriched fertilisers and from growth media.

The roots of different plants can be compatible with the same species of mycorrhizal fungi and be linked to one another by a common mycorrhizal network (CMN) in either intra- or inter-specific combinations (Newman, 1988; Newman *et al.*, 1992; 1994). One possible consequence of the existence of these links is to provide a pathway for nutrient movement between adjacent plants. NO_3^- is highly mobile and is readily transported towards the plant roots by mass flow; while NH_4^+ is adsorbed to soil colloids and transported towards the plant roots primarily by diffusion (Nommik and Vahtras, 1982; Brady and Weil, 1996). It is therefore possible that mycorrhizal hyphae play a greater role in the transport of NH_4^+ than of NO_3^- towards plant roots, except in water-deficient soil (Tobar *et al.*; 1994a, b; Wu *et al.*, 1999). In this way, N is thought to move from the N-rich donor to the N-poor receiver through the hyphae without entering the soil solution, the direction of the nutrient flow being determined by a source-sink relationship (Bethlenfalvay *et al.*, 1991). The N-concentration difference in the two plants provides the driving force for N translocation (Frey and Schuepp, 1993).

Almost all ECM fungi can assimilate NH_4^+ (Jongbloed *et al.*, 1991), but only a few can efficiently reduce NO_3^- (Scheromm *et al.*, 1990). ECM mycelial strands have been shown to transport P over several meters and ECM hyphae may extend for far greater distances into soil than AM hyphae do (Finlay and Read, 1986b). AM fungi possess the enzymes required for the assimilation of both NH_4^+ (Smith *et al.*, 1985)

and NO_3^- (Ho and Trappe, 1975). NH_4^+ is generally assimilated via the GS-GOGAT pathway in AM associations (Cliquet and Stewart, 1993; Johansen *et al.*, 1996), whereas it is metabolized through three different pathways depending on the type of ECM (Martin and Botton, 1993; Martin and Plassard, 2001), *viz.*, (1) GS occurs in the fungal sheath while GOGAT occurs in the mycorrhizal root in *Fagus*, (2) both GS and GDH exist in the sheath and the extraradical mycelium of *Picea*, respectively, (3) the GS-GOGAT pathway operates in the mycorrhizae with *Pisolithus tinctorius*.

N-transfer has been studied in both AM and ECM fungi infected plants. N-transfer from legumes to cereals has been observed through a common VA mycorrhizal network and shown to be enhanced by mycorrhizal hyphae. N-transfer, ranging from 0.05% to 45%, has been detected with NH_4^+ as the N source (Ames *et al.*, 1983; van Kessel *et al.*, 1985; Haystead *et al.*, 1988; Barea *et al.*, 1989; Eissenstat, 1990; Bethlenfalvay *et al.*, 1991; Frey and Schuepp, 1992; Azcon-Aguilar *et al.*, 1993; Johansen and Jensen, 1996). N-transfer, ranging from 0.1 to 40% with NO_3^- as the N source, has also been reported (Frey and Schuepp, 1993; Ikram *et al.*, 1994; Martin *et al.*, 1995; Martensson *et al.*, 1998).

Until now there have been only two reports of N-transfer between ECM plants. Arnebrant *et al.* (1993) found that 5 to 15% of the $^{15}NH_4^+$ in *Pinus contorta* was transferred from symbiotically fixed N by *Alnus glutinosa*, when *P. contorta* and *A. glutinosa* were connected by a common ectomycorrhizal mycelium of *Paxilus involutus*. More recently, Ekblad and Huss-Danell (1995) indicated that as much as 9.0% of the fixed N (labeled with $^{15}NH_4NO_3$) was transferred from *Alnus incana* to *Pinus sylvestris*, when *P. sylvestris* was N starved and colonised by *Paxilus involutus*. However, there is no report of N-transfer between ECM plants with NO_3^- as an external N source.

It is clear that an N_2-fixing plant can serve as an N-donor to a non-N_2-fixing plant (N-receiver) through N-transfer and that this one-way or unidirectional N-transfer can be mediated by mycorrhizal hyphae. The transfer is probable not only within ECM and AM plants themselves, but also between ECM and AM plants. However, data comparing the differences of their transfer capacities for NH_4^+ or NO_3^- are

lacking. Furthermore, most N-transfer studies to date have been limited to unidirectional transfer from an N_2-fixing plant to a non-N_2-fixing plant. However, differences in N nutritional requirements between donor and recipient may result in shifting the direction of N-transfer from the non-N_2-fixing plant to the N_2-fixing one. Brophy *et al.* (1987) pointed out that N from grasses could be transferred to associated legumes, and Tomm *et al.* (1994) reported that N-transfer could be bidirectional between bromegrass and lucerne, but such transfer was not very significant under N-limited growing conditions. Johansen and Jensen (1996) reported a very low, not statistically secured 0.3% N-transfer from the non-legume barley to the legume pea via AM hyphae. More recently, Hogh-Jensen and Schjoerring (2000) demonstrated that approximately 8% of the above-ground N in clover was derived from ryegrass. However, the VA mycorrhization status was not examined for either the N_2-fixing plant or the non-N_2-fixing one. In other words, it is not clear that VA mycorrhizae were involved in these observations. Moreover, intensive water movement under field conditions would unavoidably increase interspecific nutrient translocation between plants. Furthermore, no study on either one-way or two-way N-transfer between Australian native trees has been reported.

The following scenario may exist in nature for plants that require high N. From the point of view of evolutionary plasticity, with the addition of external N, plants may respond differently to a more nutrient rich condition. For example, an N_2-fixing plant may not exploit its capacity to fix atmospheric N_2, but may use the readily available N, especially in the early period of establishment, and thus may become relatively N-poor (= receiver). Plants adapted to nutrient poor conditions, especially those with an evolving capacity to living in nutrient-impoverished environments, such as many native Australian plants, may become relatively N-rich (= donor). If such plants become neighbours, they may alter their donor and receiver behaviour and N-transfer may occur from the non-N_2-fixing plant to the N_2-fixing plant. To our knowledge, neither one-way nor two-way N-transfer between Australian native plants has been investigated. Therefore, the studies reported in this chapter were conducted to clarify the occurrence and significance of bidirectional N-transfer between the non-leguminous actinorhizal N_2-fixing *Casuarina* and the non-N_2-fixing *Eucalyptus* via ectomycorrhizal hyphae of *Pisolithus tinctorius*, using different inorganic N sources.

3.2 Experimental Design

In general, investigations of N-transfer using $^{15}NH_4^+$ or $^{15}NO_3^-$, especially from legumes to non-legume cereals, have focused on AM infected plants (Chalk, 1996a, b, 1998; Smith and Read, 1997). Only two $^{15}NH_4^+$ transfer studies have been reported in ECM infected plants, *viz.*, from the actinorhizal N_2-fixing *Alnus* to the non-N_2-fixing *Pinus* (Arnebrant *et al.*, 1993; Ekblad and Huss-Danell, 1995). These studies showed that N-transfer occurred from the N_2-fixing plants to the non-N_2-fixing ones, and that N-transfer appeared to be mediated by mycorrhizal hyphae. Almost all these N-transfers were from the nodulated mycorrhizal N_2-fixing plants to the non-N_2-fixing ones, and none of these studies could distinguish how much transferred N was derived from the contribution of symbiotically fixed N by the N_2-fixing plant. Furthermore, only a couple of investigations showed a potential bidirectional N-transfer between non-mycorrhizal legumes and grasses (Brophy *et al.*, 1987; Tomm *et al.*, 1994; Hogh-Jensen and Schjoerring, 2000), and so far no plausible investigation of two-way N-transfer has been reported between trees either with or without ectomycorrhizal hyphae. That is to say, it is not clear whether N-transfer occurs bidirectionally, especially whether N-transfer could occur from a non-N_2-fixing to an N_2-fixing tree. If it could, bidirectionally transferred N might decrease net N transfer between plants in their N cycling process.

Also, N-transfer between ECM infected plants with NO_3^- as the external N source has not been investigated. Chemical properties and their behaviour in soils are quite different for ammonium ($^{15}NH_4^+$) and nitrate ($^{15}NO_3^-$). Therefore, not only $^{15}NH_4^+$ but also $^{15}NO_3^-$ should be used to investigate N-transfer and to compare with any N-transfer difference in ECM infected plants, due to the different preference for uptake and assimilation by plants between $^{15}NH_4^+$ and $^{15}NO_3^-$.

Two widely distributed Australian native trees, *Casuarina cunninghamiana* and *Eucalyptus maculata*, were chosen as N-donor/N-receiver pairs in experiments (Table 3.1) designed to investigate bidirectional N-transfer between them and the role of ectomycorrhizal fungus in this transfer:

Experiments I and II: bidirectional N-transfer between 6-months-old *Casuarina* and *Eucalyptus*: with $(^{15}NH_4)_2SO_4$ **(I)** and $K^{15}NO_3$ **(II)**;

Experiments III and IV: bidirectional N-transfer between 12-months-old *Casuarina* and *Eucalyptus*: with $^{15}NH_4NO_3$ **(III)** and $NH_4{}^{15}NO_3$ **(IV)**.

Table 3.1. Pairing of *Casuarina cunninghamiana* and *Eucalyptus maculata* to identify N-transfer between species. Both N-donor and N-receiver were fed continuously with external ^{14}N from transplanting. ^{15}N was supplied to the N-donor only 4 weeks before harvesting; the N-receiver was deprived of N at the same time.

N-donor		N-receiver	Code
A. N-transfer from *_Casuarina_ to _Eucalyptus_			
Pair 1: *$Casuarina_{control}$	+	$Eucalyptus_{control}$	*C1→E1
Pair 2: *$Casuarina_{ectomycorrhiza}$	+	$Eucalyptus_{control}$	*C2→E2
Pair 3: *$Casuarina_{ectomycorrhiza}$	+	$Eucalyptus_{ectomycorrhiza}$	*C3→E3
Pair 4: *$Casuarina_{Frankia}$	+	$Eucalyptus_{ectomycorrhiza}$	*C4→E4
Pair 5: *$Casuarina_{Frankia+ectomycorrhiza}$	+	$Eucalyptus_{ectomycorrhiza}$	*C5→E5
B. N-transfer from *_Eucalyptus_ to _Casuarina_			
Pair 1: *$Eucalyptus_{control}$	+	$Casuarina_{control}$	*E1→C1
Pair 2: *$Eucalyptus_{ectomycorrhiza}$	+	$Casuarina_{control}$	*E2→C2
Pair 3: *$Eucalyptus_{ectomycorrhiza}$	+	$Casuarina_{ectomycorrhiza}$	*E3→C3
Pair 4: *$Eucalyptus_{ectomycorrhiza}$	+	$Casuarina_{Frankia}$	*E4→C4
Pair 5: *$Eucalyptus_{ectomycorrhiza}$	+	$Casuarina_{Frankia+ectomycorrhiza}$	*E5→C5

* labeled with ^{15}N.

3.3 Results

3.3.1 *Biological N_2-fixation in Casuarina plants*

When NH_4^+, NO_3^- alone or NH_4^+ plus NO_3^- was the external N source, both shoot $\delta^{15}N$ and percentage of biological nitrogen fixation ($\%N_{BNF}$) in *C5* differed significantly from *C4* for both 6-months and 12-months old *Casuarina* plants (Table 3.2). Mycorrhization was developed either originally from the dual *Frankia*/mycorrhizal indoor incubation in *C5* or afterwards through the mycorrhizal partner *Eucalyptus* in *C4* during growth in the glasshouse. $\%N_{BNF}$ in 12-months-old

plants was comparatively higher than in 6-months-old plants. However, no significant difference in specific nodule activity was found between the treatments *C5* and *C4*. Also, timing of mycorrhization had no significant impact on the amount of fixed N per unit dry nodule. These results indicated that an earlier mycorrhization development in actinorhizal *Casuarina* plants could have a significant effect on both its biological N fixation capacity and the total amounts of N_2-fixed due to greater nodule production (Insets in Figure 3.9).

Table 3.2. $\delta^{15}N$ values, $\%N_{BNF}$ and specific nodule activity (mg N fixed/mg nodule dry weight) in 6-months and 12-months old *Casuarina* plants. The plants were fed continuously with external ^{14}N from transplanting [Means ± SE, n = 9; different letter (a, b) signifies difference at P = 0.05].

N-source▲	Age◆	$\delta^{15}N$		% N_{BNF}		Specific nodule activity	
		C4	*C5*	*C4*	*C5*	*C4*	*C5*
$(NH_4)_2SO_4$	6	-0.06b	-0.61a	23.89±3.29b	29.98±2.36a	0.0208±0.0003a	0.0236±0.0008a
KNO_3	6	-0.56b	-0.89a	27.07±1.64b	30.64±1.62a	0.0215±0.0027a	0.0226±0.0016a
NH_4NO_3	12	-0.05b	-0.46a	35.99±2.39b	39.78±2.80a	0.0183±0.0028a	0.0177±0.0018a

▲needles for $\delta^{15}N$ (‰) analysis harvested before labeling with ^{15}N, ◆months. $\delta^{15}N$ = –6.44‰ for nodulated *Casuarina* cultivated in an N-free medium.

3.3.2 *Formation of common mycorrhizal networks*

Plants in the control pairings retained their non-mycorrhizal status (Pairs No. 1 in Figure 3.1a-h). All plants aseptically incubated with *Pisolithus tinctorius* indoors in the four experiments maintained and developed further their mycorrhizal colonisation after 6-months or 12-months of growth in the glasshouse. The originally non-infected plants were also well colonized through their respective mycorrhizal partners by the end of the experiments (Figure 3.1; e.g. pairs 2 and 4). Thus the status of the mycorrhization virtually became the same between pairs 2 and 3, and between pairs 4 and 5, indicating that a common mycorrhizal linkage had been established either from *Casuarina* to *Eucalyptus*, or from *Eucalyptus* to *Casuarina*.

Mycorrhization was extremely significant between the non-mycorrhizal and the mycorrhizal plants in both *Casuarina* and *Eucalyptus*; and *Eucalyptus* had almost

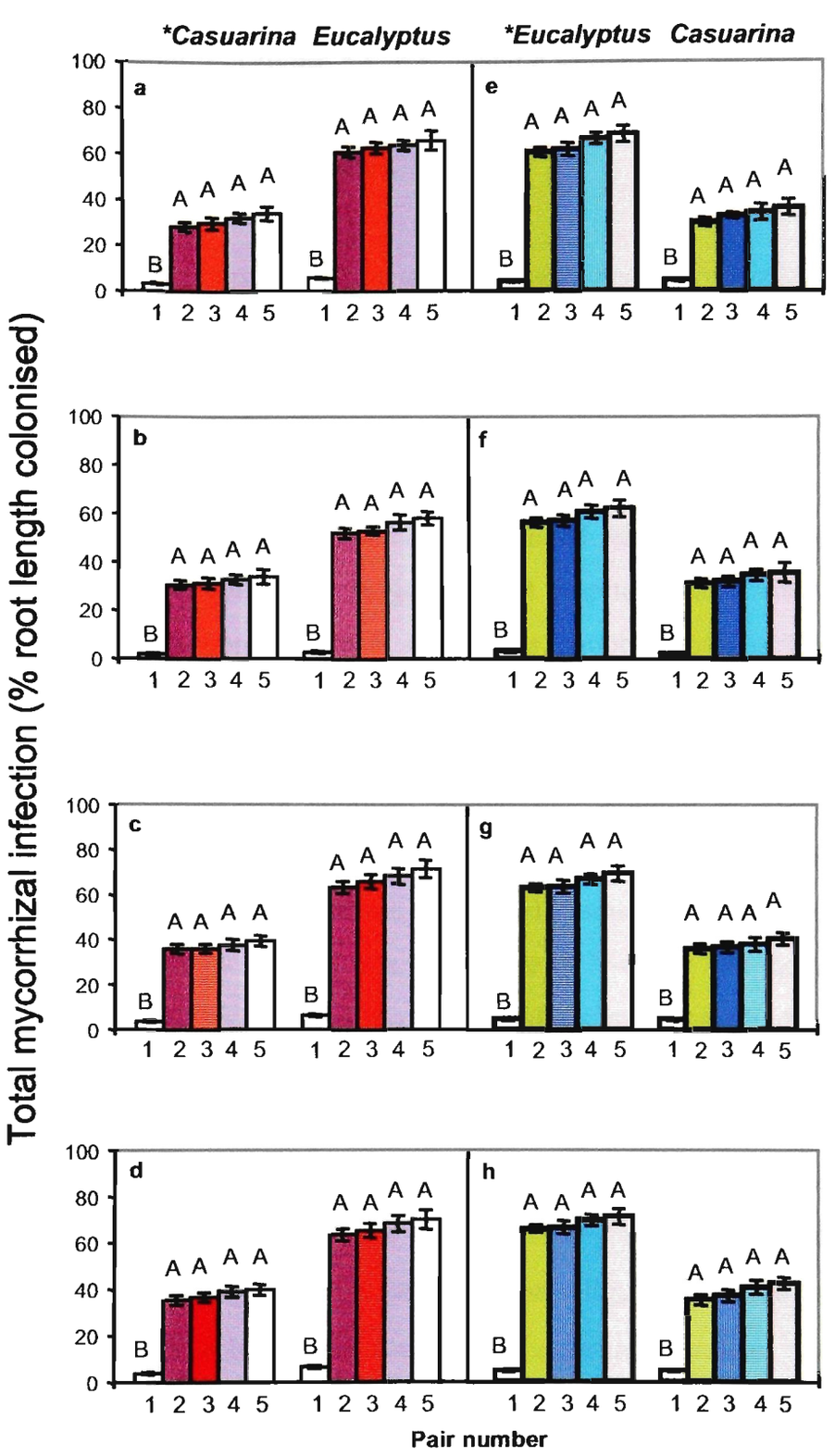

Figure 3.1. Root colonisations of **Casuarina cunninghamiana* (N-donor) and *Eucalyptus maculata* (N-receiver) (a-d), and **Eucalyptus maculata* (N-donor) and *Casuarina cumminghamiana* (N-receiver) (e-h). [Means ± SE, n = 9, different letter (A, B) signifies difference at P = 0.01; *labeled with ^{15}N].

twice the colonisation of *Casuarina* (Figure 3.1). However, root infection was not very different in the older plants (Figure 3.1c-h vs a-f) or with different external inorganic N sources (Figure 3.1, e.g., a and e vs b and f). No significant mycorrhizal colonisation difference was found between the nodulated mycorrhizal and the non-nodulated mycorrhizal casuarinas.

These results demonstrated the successful establishment of mycorrhizal colonisation and the formation of a common ectomycorrhizal network between the two species.

3.3.3 *Mycorrhizal colonisation and dry matter production*

Shoot biomass was highly positively correlated with total mycorrhizal infection in both N-donors (Figure 3.2). In general and despite approximately only half of the root colonisation compared with *Eucalyptus*, shoot biomass in *Casuarina* benefited significantly more from mycorrhization than in *Eucalyptus* (Figure 3.2B-H), especially when it served as the N-receiver. The exception was Experiment I where NH_4^+ was continuously supplied as the sole external N source (Figure 3.2A). *Eucalyptus* did not show much increase in shoot biomass over the non-inoculated control in any pairing irrespective of external N source (Figure 3.2A-H), and this was much more obvious when it was in the role of N-donor. On the other hand, all the nodulated mycorrhizal *Casuarina* plants had a significantly higher shoot dry matter yield than the non-nodulated mycorrhizal ones. Similarly, *Eucalyptus* growing with the dual *Frankia*/mycorrhizal fungi infected casuarinas accumulated more shoot biomass than those with single mycorrhizal fungi infected *Casuarina* partners. Another distinction was that the N-receivers of *C4* and *C5* accumulated relatively much more shoot dry matter when NO_3^- alone (Figure 3.2F) rather than NH_4^+ or the combined NO_3^- + NH_4^+ was used as the external N-source. There were no root biomass differences among pairs 2 to 5 of the 12-months-old N-donor *Eucalyptus*, when supplied continuously with the combined $NH_4^+NO_3^-$.

Correlation analysis also revealed a moderate relationship between root biomass and root mycorrhizal colonisation (Figure 3.3). Mycorrhization had a significant effect on root dry matter production of both *Casuarina* and *Eucalyptus*. Nodulated mycorrhizal *Casuarina* had the highest root biomass; this was also generally the case

for the paired *Eucalyptus* (Figure 3.3). On the other hand, root biomass in the 6-months-old plants (Figure 3.3A-B and E-F) with external $(NH_4)_2SO_4$ or KNO_3 was different, compared to the relatively similar root biomass production with the combined $^{15}NH_4{}^{14}NO_3^-$ or $^{14}NH_4{}^{+15}NO_3^-$.

The total biomass values and the relationship between biomass and mycorrhization reflected those of individual root and shoot production. The correlation between total biomass (shoot plus root) and total root mycorrhizal infection was positively high for both species, and the regression lines were much sharper in *Casuarina* than in *Eucalyptus* (Figure 3.4). With only about a third of the root mycorrhizal infection, whether as N-donor or N-receiver, *Casuarina* always benefited more from mycorrhization, especially when nodulated and as the 6-months-old N-receiver, except when NH_4^+ was the sole N source (Figure 3.4A). This is probably the inhibition of growth performance by the accompanying SO_4^{2-} and mycorrhization is secondarily important for *Casuarina*. Meanwhile, no root biomass differences were found in pairs 2 to 5 of the 12-months-old N-donor *Eucalyptus*, when the plants were continuously fed with the combined $NH_4^+NO_3^-$.

In summary, no matter whether NH_4^+, NO_3^- alone or NH_4^+/NO_3^- combined could be accessed by both the 6-months and the 12-months-old plants, the above data indicated that (1) biomass production in both *Casuarina* and *Eucalyptus* was positively correlated with their root mycorrhizal colonisation. Either in casuarinas or their corresponding partner eucalypts, biomass differed generally in the following pattern: the dual nodulated mycorrhizal > the sole mycorrhizal > the non-mycorrhizal pairing; (2) *Casuarina* received a growth benefit either from nodulation or mycorrhization alone, and it was the combination of *Frankia* and mycorrhizal fungus that had the greatest effect on *Casuarina* growth performance; (3) the dual *Frankia*/mycorrhizal *Casuarina* not only had a significant effect on its own growth performance, but also generally contributed substantial benefit to its adjacent neighbor *Eucalyptus*, if the latter was connected to these nodulated mycorrhizal *Casuarina* by a common mycorrhizal network.

Casuarina can grow well with approximately one-half the root mycorrhizal infection

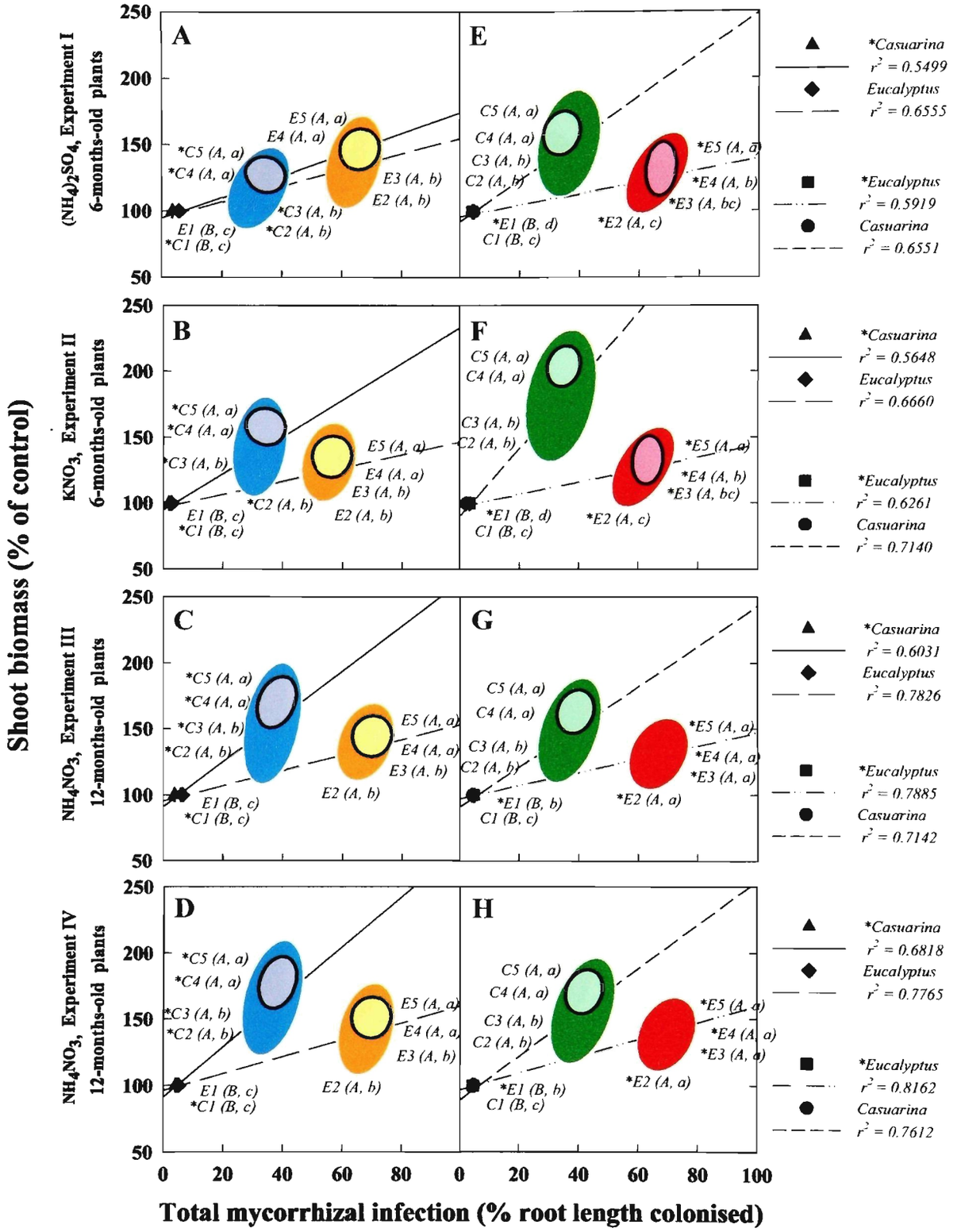

Figure 3.2. Relationships between root mycorrhizal colonisation and shoot biomass production of ***Casuarina*** (N-donor) and ***Eucalyptus*** (N-receiver) (A-D); and ***Eucalyptus*** (N-donor) and ***Casuarina*** (N-receiver) (E-H). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x*, *y* parameters, respectively].

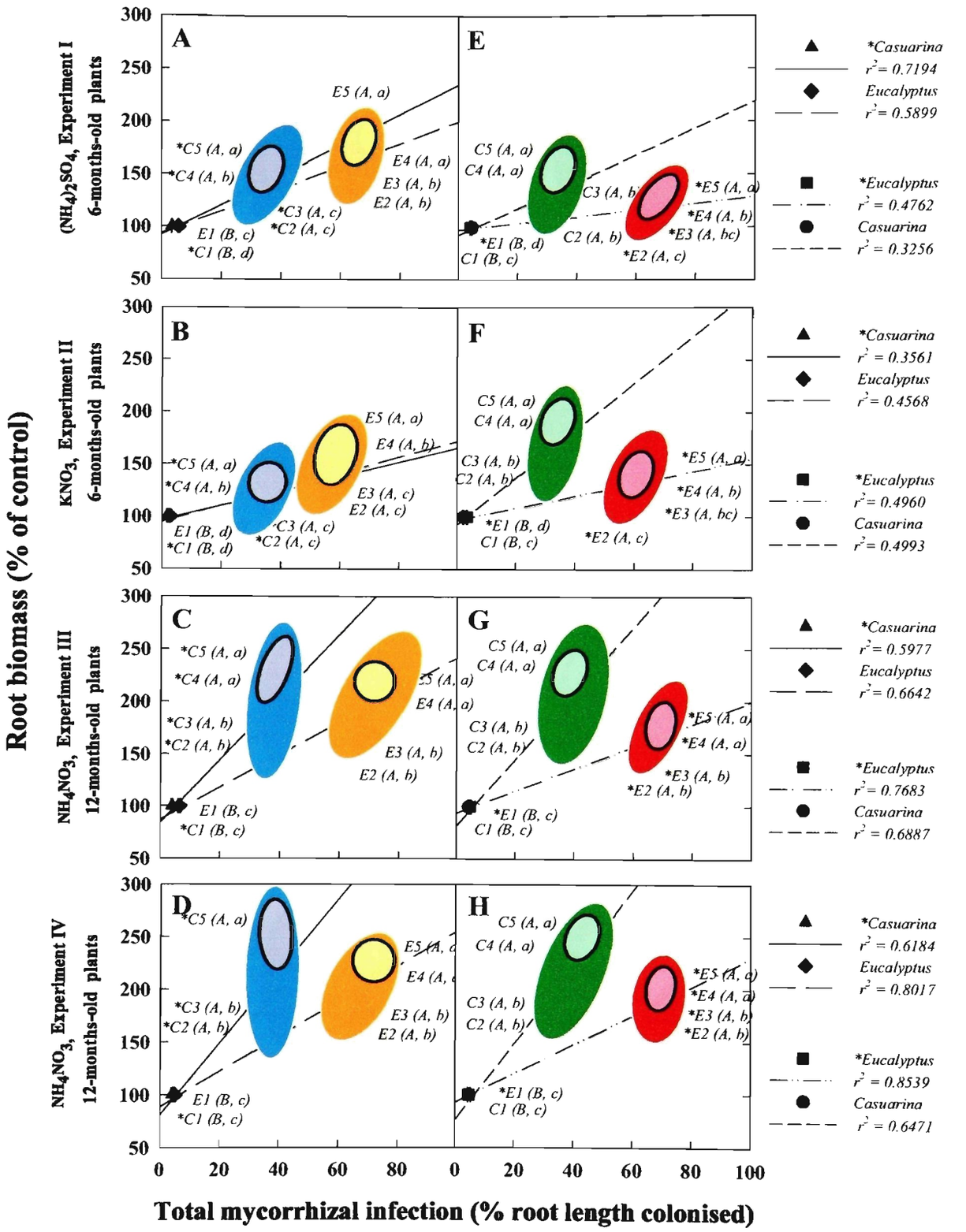

Figure 3.3. Relationships between root mycorrhizal colonisation and root biomass production of ***Casuarina*** (N-donor) and *Eucalyptus* (N-receiver) (A-D); and ***Eucalyptus*** (N-donor) and *Casuarina* (N-receiver) (E-H). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

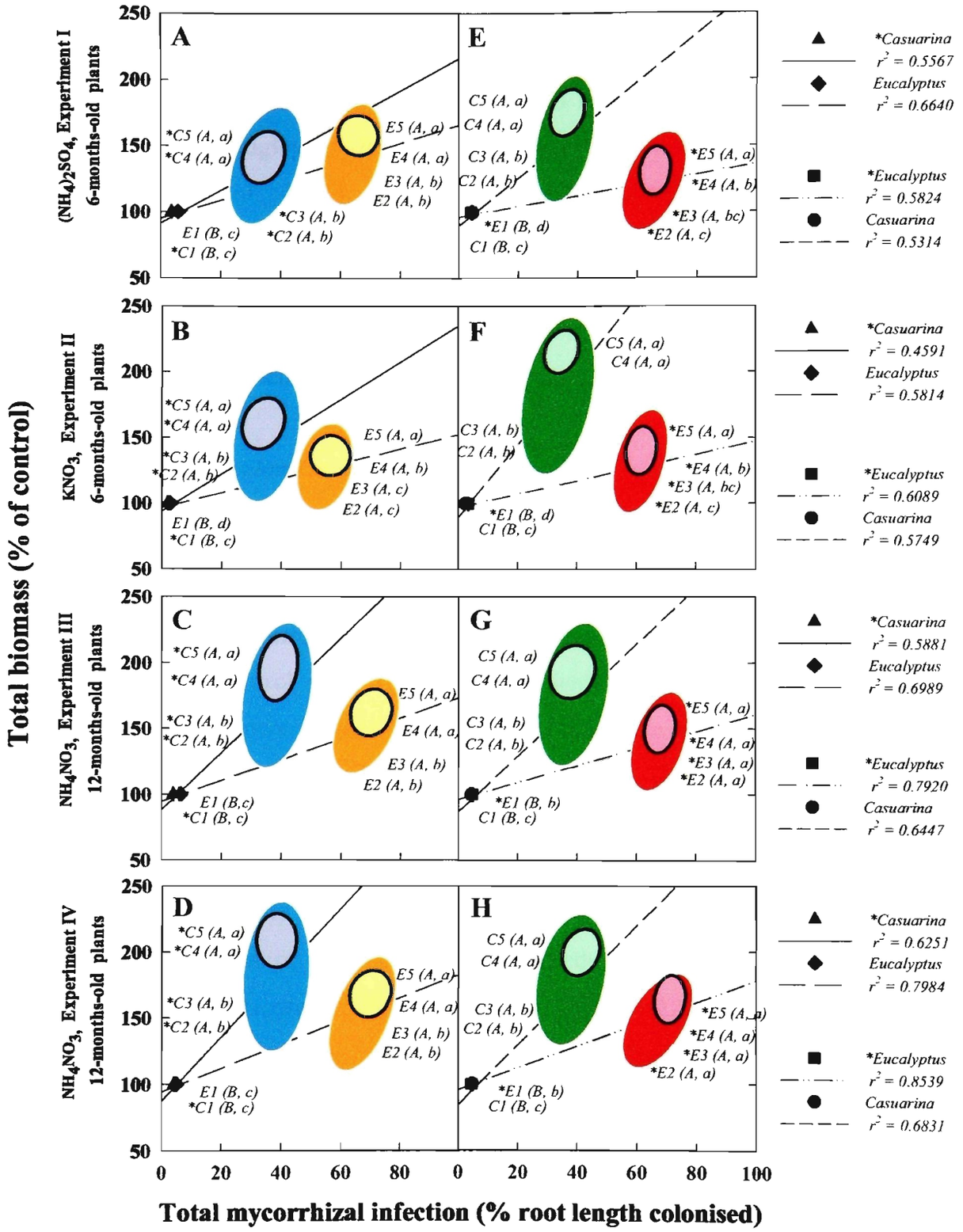

Figure 3.4. Relationships between root mycorrhizal colonisation and total biomass production of ***Casuarina*** (N-donor) and *Eucalyptus* (N-receiver) (A-D); and ***Eucalyptus*** (N-donor) and *Casuarina* (N-receiver) (E-H). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

seen in *Eucalyptus* and achieve maximum biomass accumulation, when dual nodulated/mycorrhizal. This is because it is an N_2-fixing plant with *Frankia* and mycorrhization is of secondary importance to N acquisition in this species. Either physiologically or ecologically, *Casuarina* requires relatively higher amounts of N for optimal biomass accumulation/growth which depend on both N_2-fixation and mycorrhization (Midgley *et al.*, 1983; Subbarao and Rodriguez-Barrueco, 1995; Pinyopusarerk *et al.*, 1996). In contrast, *Eucalyptus* requires much less N by comparison (Dell *et al.*, 1995; Williams and Woinarski, 1997), but benefits relatively more from mycorrhization, which is the primary and the easiest way to acquire the little N it needs for its growth performance. More N fertilisation, in the case of being the N-donor, did not benefit *Eucalyptus* directly; it was probably even inhibitory.

3.3.4 *Mycorrhizal colonisation and N content*

Shoot N accumulation was positively related to root total mycorrhizal infection (Figure 3.5). Significant shoot N differences were ranked as follows for *Casuarina*: the dual *Frankia*/mycorrhizal pairs 4 and 5 > the sole mycorrhizal pairs 2 and 3 > the non-mycorrhizal control pairs 1 (Figure 3.5), while the 6-months-old *Casuarina* accumulated much more N when it was the N-receiver than when it was the N-donor (Figure 3.5E, F vs. 3.5A, B). This was also the case for *Eucalyptus* treatments that showed a corresponding N accumulation trend with *Casuarina* partners. Meanwhile, the 6-months-old *Casuarina* and *Eucalyptus* (Figure 3.5A, B, E and F) in pairs 5 exhibited a significantly higher N content than that in pairs 4, no matter whether it acted as the N-donor or the N-receiver and whether NH_4^+ or NO_3^- was the sole external N-source. However, this was not true for the 12-months-old *Casuarina* (Figure 3.5C and D) or *Eucalyptus* (Figure 3.5G and H) between pair 4 and pair 5.

A good correlative relationship between root N accumulation and mycorrhization was also found for both *Casuarina* and *Eucalyptus* (Figure 3.6). In general, root N accumulation showed very similar, but more enhanced trends than those seen in root biomass yield. Especially pronounced was the influence of N_2-fixation in the N-receiver *Casuarina* with NO_3^- as the N source (Figure 3.6 F) where root N content was 3-fold higher than in the control *Casuarina* or indeed all of the N-donor *Eucalyptus*. Both species showed significant root N accumulation as this order: the

nodulated mycorrhizal pairs 4 and 5 > the non-nodulated mycorrhizal pairs 2 and 3 > the non-mycorrhizal control pairs 1 (Figure 3.6). Meanwhile, N accumulation in pairs 5, the 6-months-old *Casuarina* (Figure 3.6B) and *Eucalyptus* (Figure 3.6E, F), and the 12-months-old *Eucalyptus* (Figure 3.6C, D), differed significantly from that in pairs 4. The most interesting results for root N content came from the N-receiver *Eucalyptus* which accumulated relatively much more N with both NH_4^+ and NO_3^- as N source and when the N-donor *Casuarina* was nodulated (Figure 3.6C, D).

Total N content, averaged over the measured shoot and root values, correlated moderately well with root mycorrhization, and the regression lines were much sharper in *Casuarina* than in *Eucalyptus* (Figure 3.7). As a general rule, the nodulated and mycorrhizal *Casuarina* and their mycorrhizal partner *Eucalyptus*, had significantly higher total N contents than non-nodulated but mycorrhizal counterparts. The non-nodulated but mycorrhizal plants in turn had a significantly greater total N content than the non-mycorrhizal ones. The sole mycorrhizal plants also had a significantly greater total N content than the non-mycorrhizal ones. Nitrogen contents in pairs 5 were significantly different from those in pairs 4 for both 6-months-old *Casuarina* and *Eucalyptus*, and also the 12-months-old N-receiver *Eucalyptus*, but not between pairs 4 and 5 in other 12-months-old plants. On the other hand, all the casuarinas showed better responses to mycorrhizal colonisation than *Eucalyptus* except for the single mycorrhizal treatments (Figure 3.7B) where NO_3^- was the sole external N-source. Nitrogen accumulation also responded more profoundly to mycorrhization in the 6-months-old 'N-receiver' nodulated mycorrhizal *Casuarina*, compared to the 6-months-old 'N-donor' nodulated mycorrhizal *Casuarina* with a further 4-weeks external ^{15}N supply. The opposite was true in N accumulation between the N-donor and the N-receiver eucalypts.

In summary, although root mycorrhizal infection was much greater in *Eucalyptus* than in *Casuarina*, mycorrhization had little effect on N accumulation in *Eucalyptus*. On the other hand, mycorrhization had a major effect on N accumulation in *Casuarina* though it had less mycorrhizal infection. Nitrogen content was highest in the nodulated mycorrhizal pairs, especially when nodulated casuarinas were the N-receivers. Nitrogen content also pretty much reflected biomass accumulation, the largest increases being seen in N-receiver *Casuarina* with NO_3^- as the N source.

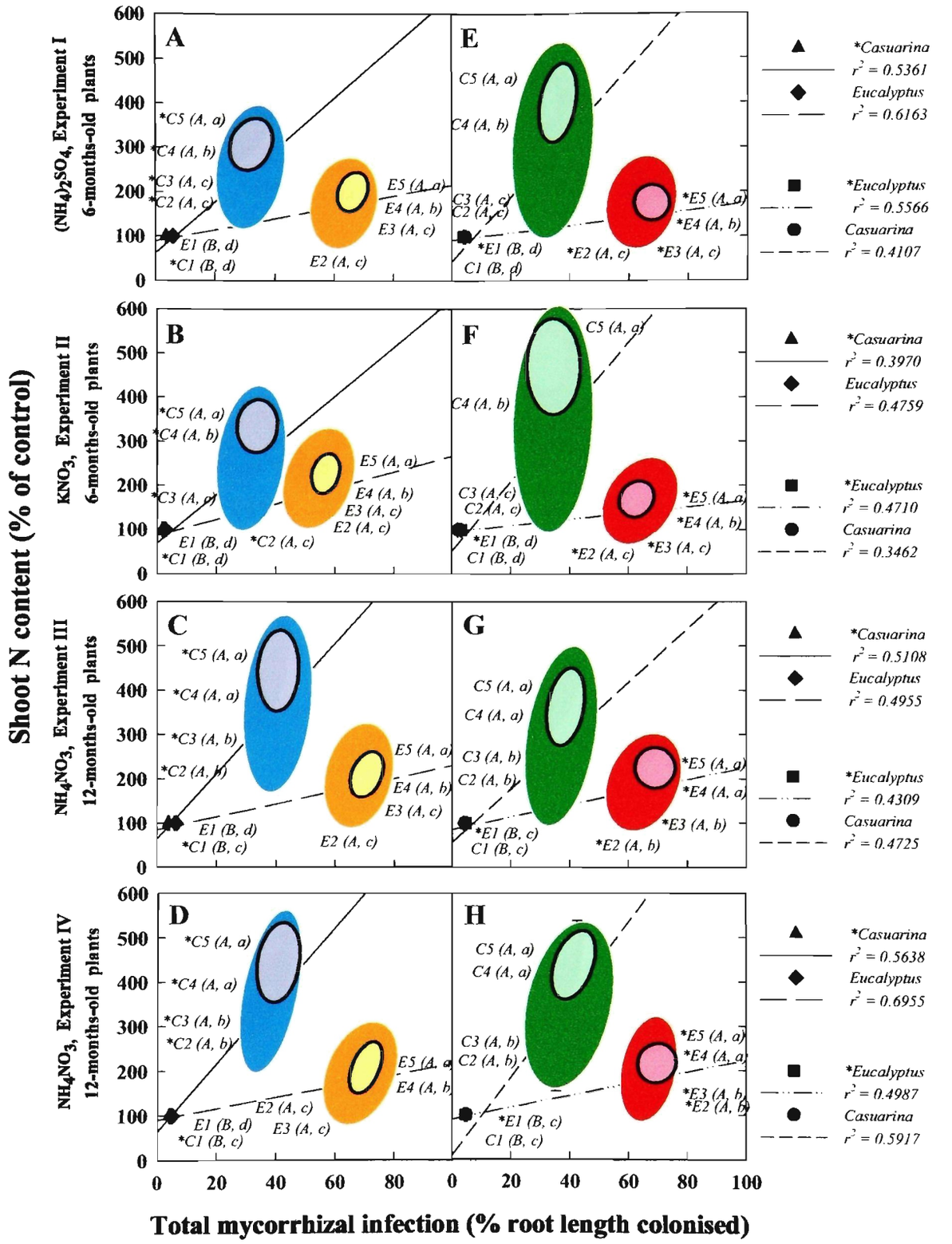

Figure 3.5. Relationships between root mycorrhizal colonisation and shoot nitrogen content of ***Casuarina*** (N-donor) and ***Eucalyptus*** (N-receiver) (A-D); and ****Eucalyptus*** (N-donor) and ***Casuarina*** (N-receiver) (E-H). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x*, *y* parameters, respectively].

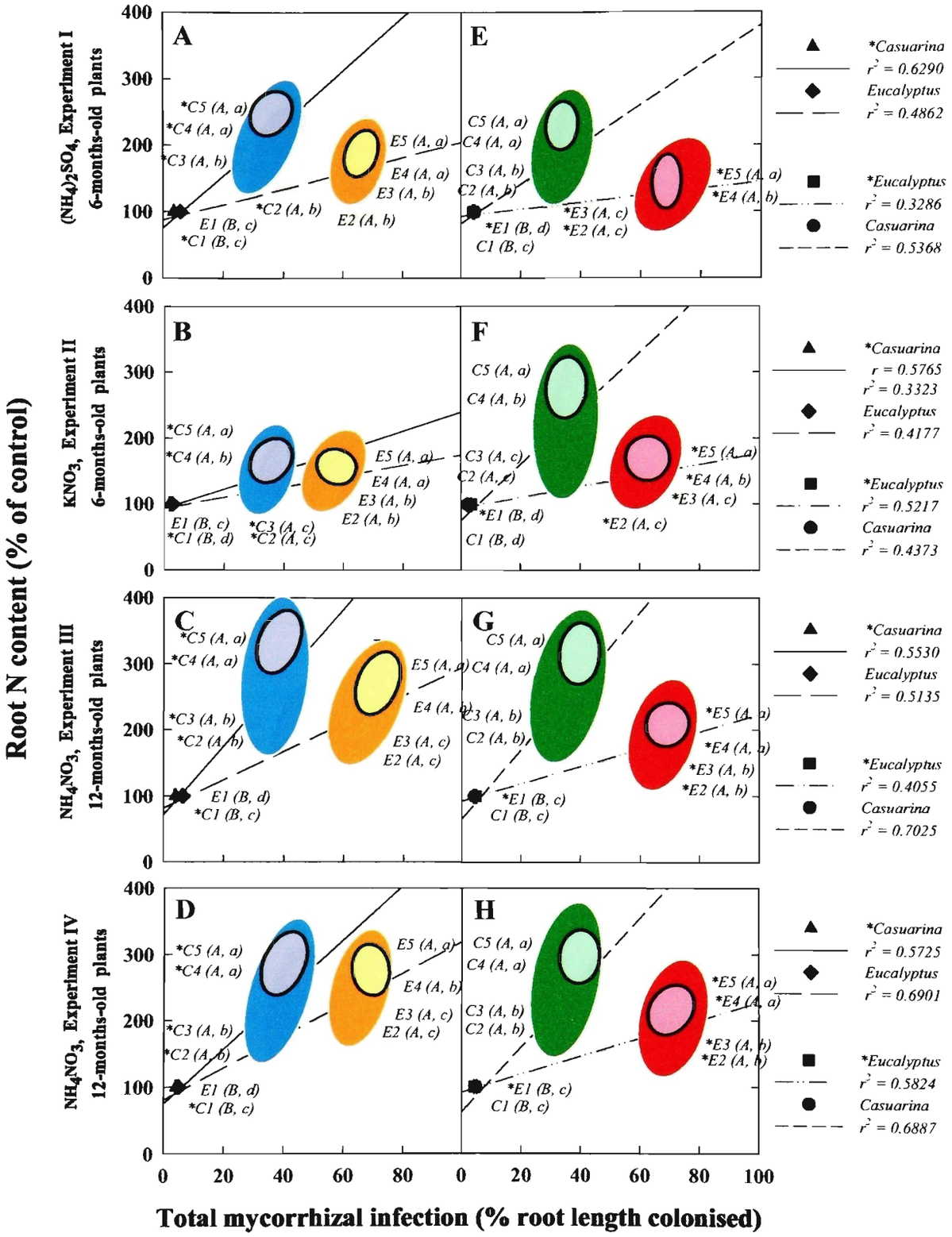

Figure 3.6. Relationships between root mycorrhizal colonisation and root nitrogen content of **Casuarina* (N-donor) and *Eucalyptus* (N-receiver) (A-D); and **Eucalyptus* (N-donor) and *Casuarina* (N-receiver) (E - H). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

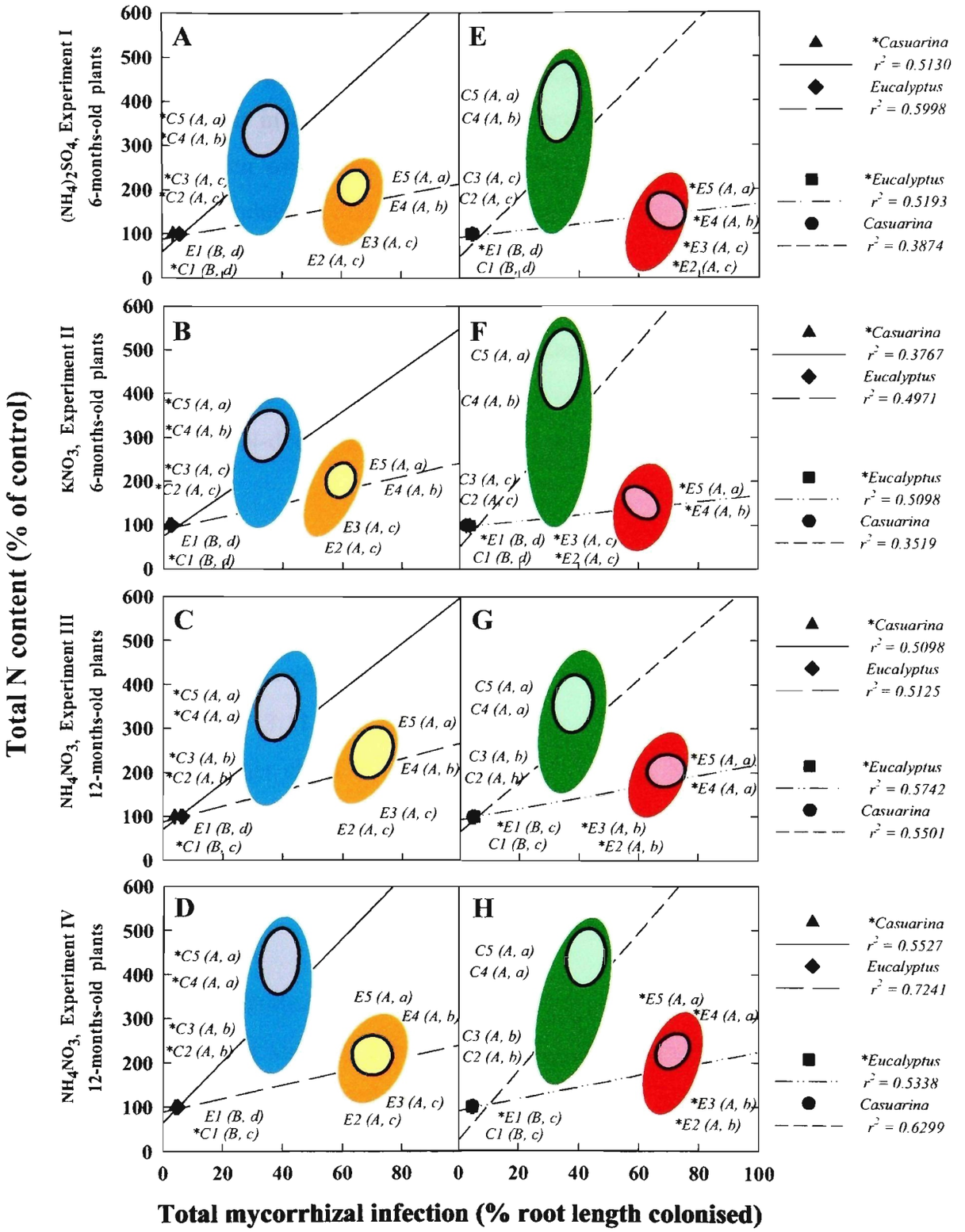

Figure 3.7. Relationships between root mycorrhizal colonisation and total niotrogen content of ***Casuarina*** (N-donor) and ***Eucalyptus*** (N-receiver) (A-D); and ***Eucalyptus*** (N-donor) and ***Casuarina*** (N-receiver) (E-H). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x*, *y* parameters, respectively].

3.3.5 *Relationship between N content and biomass production*

Consistent with results shown in Figures 3.2 – 3.7, biomass production in both species was highly positively correlated with tissue N content for shoot, root and total when biomass is plotted against total N content (Figure 3.8, 3.9 and 3.10). Biomass production in both *Casuarina* and *Eucalyptus* increased with N content. Compared with their own controls, nodulated mycorrhizal *Casuarina* had both the highest biomass production and N accumulation, especially in the case when the 6-months-old *Casuarina* was the N-receiver.

In addition, both biomass production and N accumulation of the root nodules in the **C5* N-donor and the *C5* N-receiver plants were greater than those of the root nodules in the **C4* N-donor and *C4* N-receiver plants of *Casuarina* (Insets in Figure 3.9).

3.3.6 *Relationship between shoot N concentration or content and $\delta^{15}N$ value*

$\delta^{15}N$ values in *Casuarina* were often negative and much lower than those in *Eucalyptus* (Figures 3.11 and 3.12). The lower $\delta^{15}N$ values in *Casuarina* were associated with higher shoot N concentration or N shoot N content. Both shoot N concentration and shoot N content did not vary nearly as much with different N source in the two species, but shoot concentration doubled and shoot N content were much higher in *Casuarina* than in *Eucalyptus*.

The results demonstrated that (1) shoot $\delta^{15}N$ value (‰) was closely inversely correlated with either shoot N concentration (%) or content (Log_{10} mg N/plant) for both species; (2) the nodulated mycorrhizal *Casuarina* had a significantly lower shoot $\delta^{15}N$ values than the non-nodulated mycorrhizal or the non-mycorrhizal *Casuarina*. These results were also generally true between the mycorrhizal and the non-mycorrhizal *Eucalyptus*.

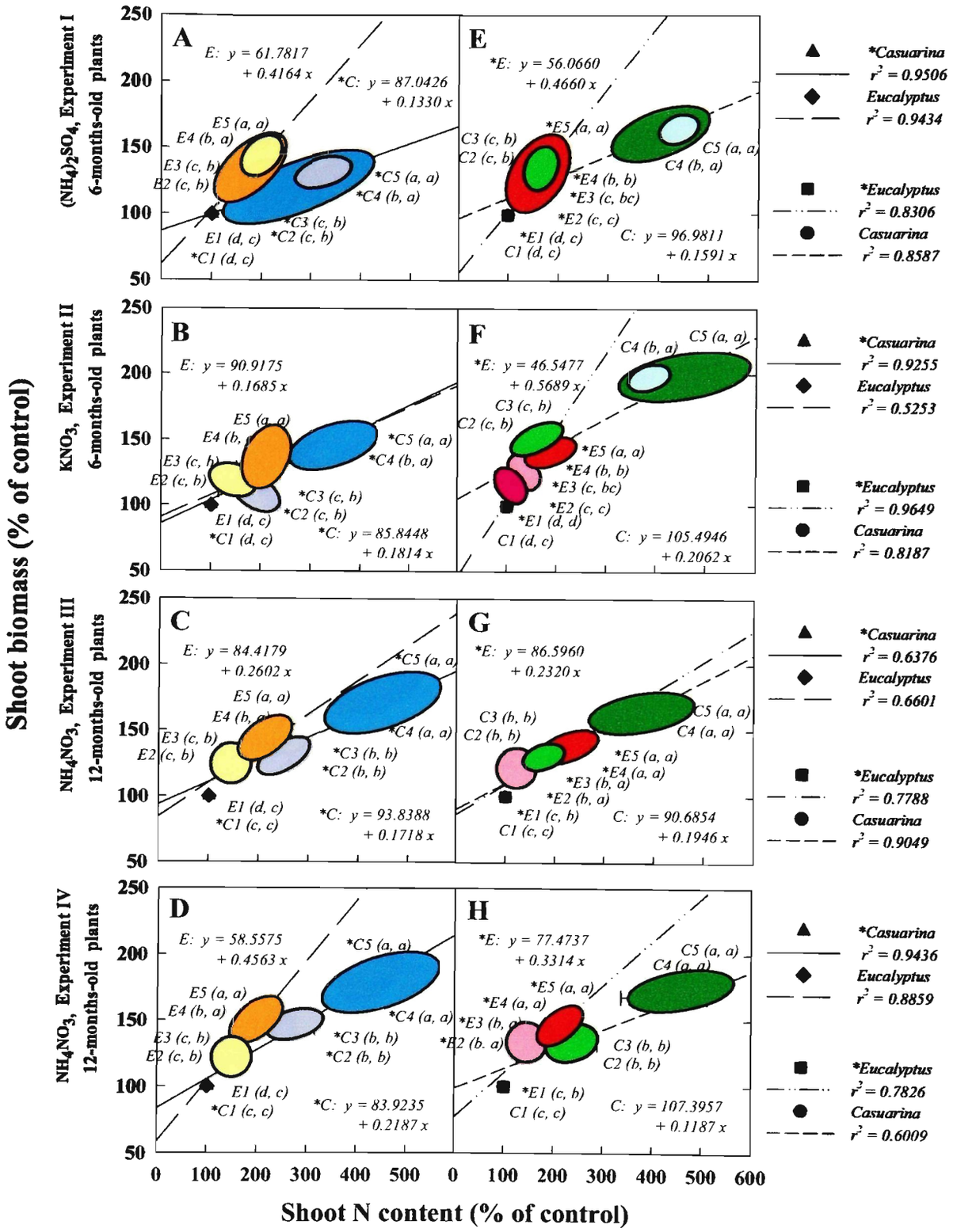

Figure 3.8. Relationships between shoot N content and shoot biomass production of **Casuarina* (N-donor) and *Eucalyptus* (N-receiver) (A - D); and **Eucalyptus* (N-donor) and *Casuarina* (N-receiver) (E - H). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x*, *y* parameters, respectively].

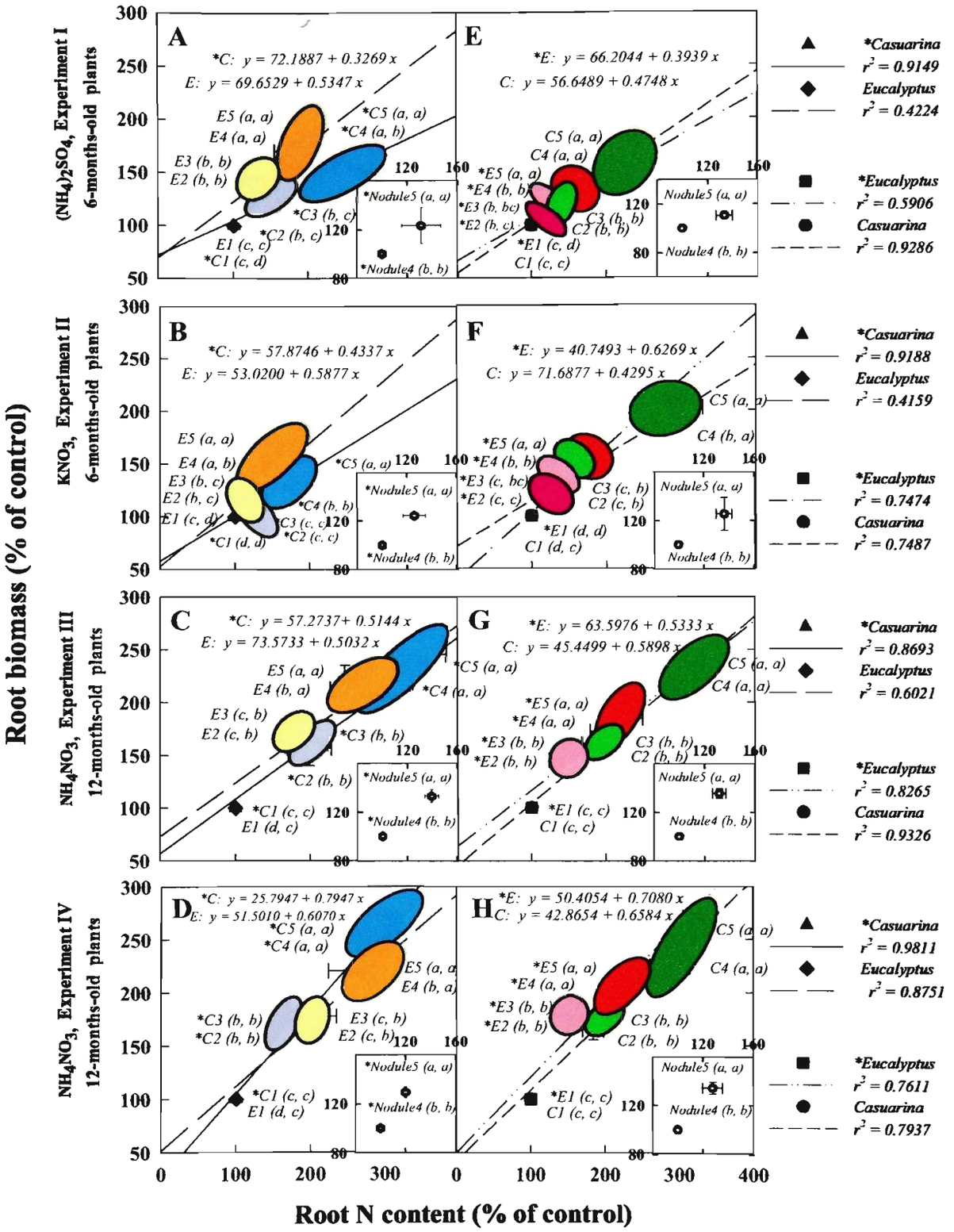

Figure 3.9. Relationships between root N content and root biomass production of **Casuarina* (N-donor) and *Eucalyptus* (N-receiver) (A - D); and **Eucalyptus* (N-donor) and *Casuarina* (N-receiver) (E - H). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x*, *y* parameters, respectively].

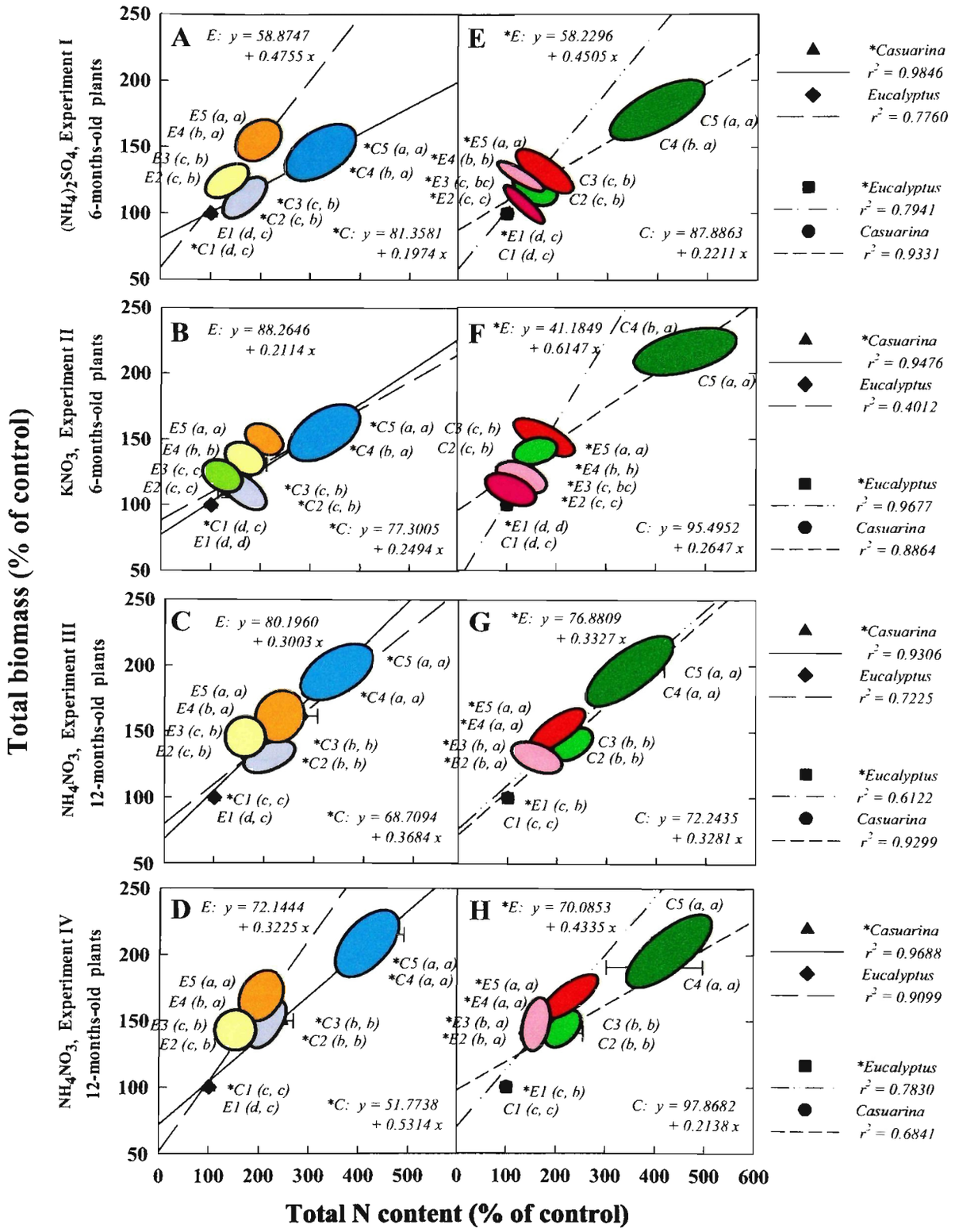

Figure 3.10. Relationships between total N content and total biomass production of **Casuarina* (N-donor) and *Eucalyptus* (N-receiver) (A-D); and **Eucalyptus* (N-donor) and *Casuarina* (N-receiver) (E-H). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

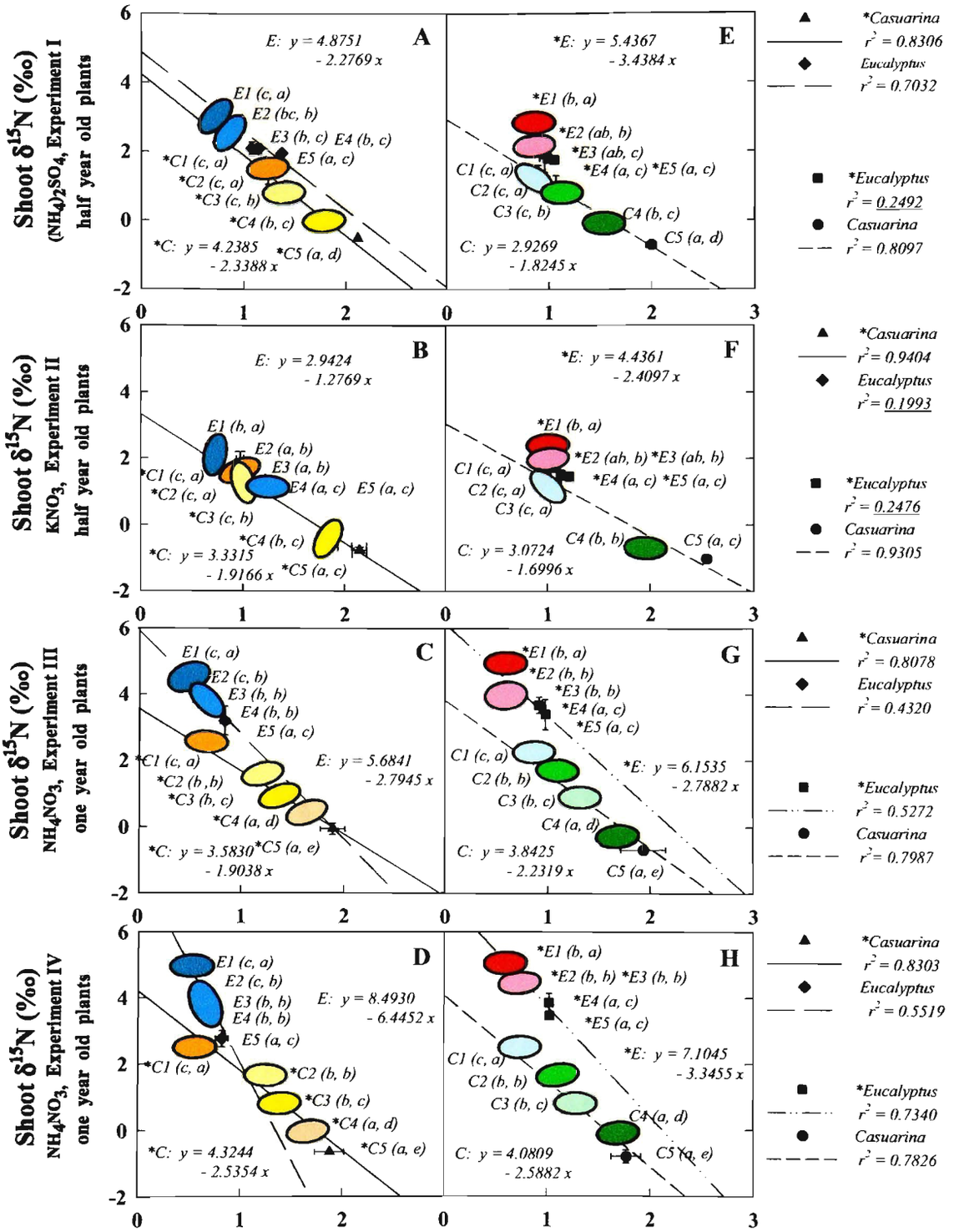

Figure 3.11. Relationships between shoot N concentration and shoot $\delta^{15}N$ value of **Casuarina* (N-donor) and *Eucalyptus* (N-receiver) (A-D); and **Eucalyptus* (N-donor) and *Casuarina* (N-receiver) (E-H). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x*, *y* parameters, respectively].

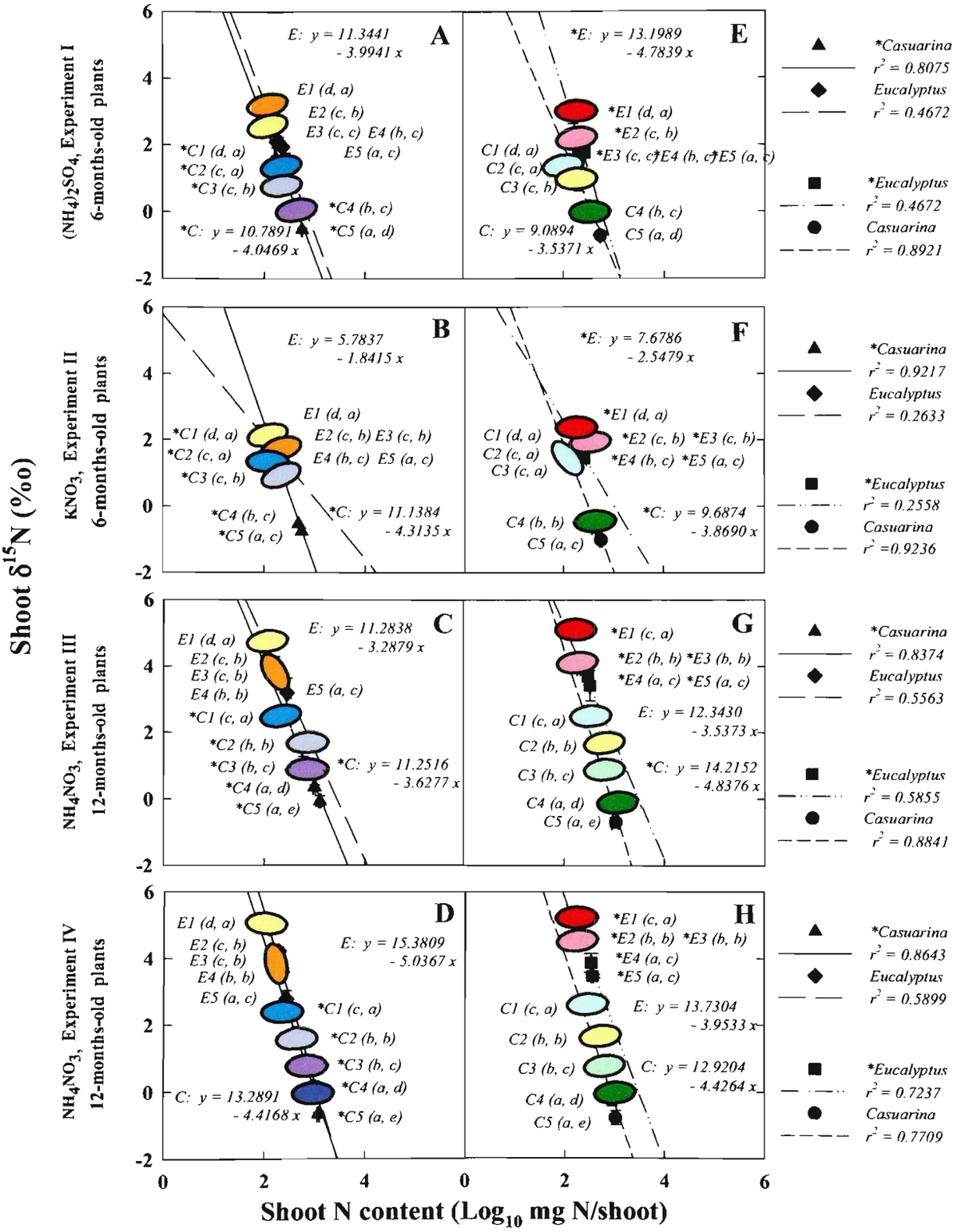

Figure 3.12. Relationships between shoot N content and shoot $\delta^{15}N$ value of ***Casuarina*** (N-donor) and ***Eucalyptus*** (N-receiver) (A-D); and ****Eucalyptus*** (N-donor) and ***Casuarina*** (N-receiver) (E-H). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x*, *y* parameters, respectively].

3.3.7 *Nitrogen transfer between Casuarina and Eucalyptus*

3.3.7.1. *$(^{15}NH_4)_2SO_4$ labeling analysis*

Table 3.3 shows that more N was transferred from *Eucalyptus* to *Casuarina* than the other way around. The results suggested, for 6 months old plants, if NH_4^+ was the sole external feeding N-source and the labeled ^{15}N was employed in the form of $(^{15}NH_4)SO_4$, that (1) N-transfer can be bidirectional either from **Casuarina* to *Eucalyptus* or from **Eucalyptus* to *Casuarina*, (2) no matter which served as the N-donor or the N-receiver, the pairings with the nodulated mycorrhizal casuarinas (**C5→E5, *E5→C5, *C4→E4, *E4→C4*) had a significantly greater N-transfer than those with the sole mycorrhizal *Casuarina* plants (**C3→E3, *C2→E2, *E3→C3, *E2→C2*), (3) either in the shoot, the root and the total, N-transfer was generally higher from **Eucalyptus* to *Casuarina* than from **Casuarina* to *Eucalyptus*,

Table 3.3. N-transfer from **Casuarina cunninghamiana* (N-donor) and *Eucalyptus maculata* (N-receiver) (A), and **Eucalyptus maculata* (N-donor) and *Casuarina cunninghamiana* (N-receiver) (B) after 6 months growth in the glasshouse. Both the N-donor and the N-receiver were fed continuously with external ^{14}N since transplanting. ^{15}N was supplied to the N-donor only 4 weeks before harvesting; the N-receiver was deprived of external N for the same period [Values are Means, n = 9, different letter (a, b) signifies difference at P = 0.05].

Treatments	N-transfer								
	% $N_{transfer}$			$N_{transfer}$ (mg/plant)			% NDFT		
	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total
ExperimentA: N-transfer from *Casuarina (N-donor) to *Eucalyptus* (N-receiver)									
C5→E5	1.38a	3.91a	2.64a	3.57a	2.80a	6.37a	3.13a	8.87a	6.00a
C4→E4	1.32a	2.39b	1.91a	2.21b	1.32b	3.53b	2.69a	5.31b	4.00b
C3→E3	1.01b	1.09c	1.06b	1.09c	0.38c	1.47c	1.83b	3.08c	2.45c
C2→E2	0.78b	0.85c	0.33b	0.76c	0.18c	0.94c	1.50b	1.49c	1.50c
C1→E1	—	—	—	—	—	—	—	—	—
ExperimentB: N-transfer from *Eucalyptus* (N-donor) to *Casuarina* (N-receiver)									
E5→C5	18.03a	36.26a	26.81a	22.73a	5.88a	28.62a	5.36a	10.05a	7.71a
E4→C4	10.10b	21.14b	14.09b	8.10b	1.60b	9.70b	2.72b	3.18b	2.95b
E3→C3	1.99c	3.04c	2.49c	0.91c	0.24c	1.15c	1.10c	1.00c	1.05c
E2→C2	0.62c	0.35c	0.51c	0.24c	0.03c	0.27c	0.44c	0.52c	0.48c
E1→C1	—	—	—	—	—	—	—	—	—

*labeled with $(^{15}NH_4)_2SO_4$.

provided that **Eucalyptus* was paired with the nodulated mycorrhizal *Casuarina* and the *Casuarina* was deprived of external N supply for a period of 4 weeks before harvesting, and (4) the amount of N-transferred was similar if the potentially N_2-fixing casuarinas were not nodulated, no matter whether the N-transfer was from **Casuarina* to *Eucalyptus* or from **Eucalyptus* to *Casuarina*.

3.3.7.2. *$K^{15}NO_3$ labeling analysis*

Labeling with $^{15}NO_3$ yielded very similar results showing that significantly more N-transfer occurred from *Eucalyptus* to *Casuarina*, especially with nodulated and mycorrhizal *Casuarina* (Table 3.4).

Table 3.4. N-transfer from **Casuarina cunninghamiana* (N-donor) and *Eucalyptus maculata* (N-receiver) (A), and **Eucalyptus maculata* (N-donor) and *Casuarina cunninghamiana* (N-receiver) (B) after 6 months growth in the glasshouse. Both the N-donor and the N-receiver were fed continuously with external ^{14}N since transplanting. ^{15}N was supplied to the N-donor only 4 weeks before harvesting; the N-receiver was deprived of external N for the same period [Values are Means, n = 9, different letter (a, b) signifies difference at P = 0.05].

Treatments	N-transfer								
	% $N_{transfer}$			$N_{transfer}$ (mg/plant)			% NDFT		
	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total
ExperimentA: N-transfer from *Casuarina (N-donor) to *Eucalyptus* (N-receiver)									
***C5→E5**	2.55a	3.04a	2.80a	5.81a	2.17a	7.98a	5.19a	11.73a	8.46a
***C4→E4**	2.80a	2.32a	2.56a	4.41a	1.38a	5.79a	5.00a	8.55a	6.78a
***C3→E3**	1.39b	1.50b	1.45b	1.08b	0.31b	1.39b	1.55b	3.46b	2.51b
***C2→E2**	1.13b	0.74b	0.94b	0.60b	0.16b	0.75b	1.06b	2.25b	1.66b
***C1→E1**	—	—	—	—	—	—	—	—	—
ExperimentB: N-transfer from *Eucalyptus (N-donor) to *Casuarina* (N-receiver)									
***E5→C5**	13.08a	15.90a	14.90a	15.88a	5.70a	21.58a	6.05a	9.19a	7.62a
***E4→C4**	4.84b	11.94b	9.39b	3.14b	2.83b	6.27b	3.23b	6.58b	4.90b
***E3→C3**	2.21c	3.70c	3.19c	0.99c	0.54c	1.53c	1.34c	2.45c	1.89c
***E2→C2**	1.16c	3.24c	2.59c	0.22c	0.32c	0.54c	0.63c	1.78c	1.20c
***E1→C1**	—	—	—	—	—	—	—	—	—

*labeled with $K^{15}NO_3$.

The results suggested, for 6 months old plants, if NO_3^- was used as the sole external N-source and the labeled ^{15}N was employed in the form of $K^{15}NO_3$, that (1) N-transfer could be bidirectional either from **Casuarina* to *Eucalyptus* or from **Eucalyptus* to *Casuarina*, (2) no matter which served as the N-donor or the N-receiver, the pairings with the nodulated mycorrhizal casuarinas (**C5→E5, *E5→C5, *C4→E4, *E4→C4*) had significantly higher N-transfer than those with the sole mycorrhizal *Casuarina* plants (**C3→E3, *C2→E2, *E3→C3, *E2→C2*), (3) either in the shoot, the root and the total, N-transfer was generally higher from **Eucalyptus* to *Casuarina* than from **Casuarina* to *Eucalyptus*, provided that **Eucalyptus* was paired with the nodulated mycorrhizal *Casuarina* and the *Casuarina* was deprived of its external N supply for a period of 4 weeks before harvesting, and (4) the amount of N-transferred was similar if the potentially N_2-fixing casuarinas were not nodulated, no matter whether the N-transfer was from **Casuarina* to *Eucalyptus* or from **Eucalyptus* to *Casuarina*.

3.3.7.3 *$^{15}NH_4NO_3$ and $NH_4{}^{15}NO_3$ labeling analysis*

The differences in N-transfer from 12-months-old *Eucalyptus* to *Casuarina* were even more pronounced and consistent across the pairings when $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ was used as the external labeling N source (Table 3.5).

3.3.7.4 *Nitrogen transfer between Casuarina and Eucalyptus: summaries*

The ^{15}N labeling results described above clearly showed that (1) N could be transferred bidirectionally between the N_2-fixing *Casuarina* and the non-N_2-fixing *Eucalyptus*, (2) no matter whether *Casuarina* or *Eucalyptus* acted as the N-donor or the N-receiver, the pairings with the nodulated mycorrhizal *Casuarinas* generally had greater N-transfer, expressed as either %N-transfer or the amount of N-transferred (mg/plant), suggesting that N-transfer was enhanced by symbiotic N_2-fixation, (3) the amounts of N-transferred (mg/plant) and the percentage of N-transfer (%) were significantly higher from *Eucalyptus* to *Casuarina,* and (4) the %NDFT (the % of N in the receiver derived from transfer) was approximately in the same range in all *Casuarina* and *Eucalyptus* pairings, from either **Casuarina* to *Eucalyptus* or **Eucalyptus* to *Casuarina*.

Table 3.5. N-transfer from **Casuarina cunninghamiana* (N-donor) and *Eucalyptus maculata* (N-receiver) (A), and **Eucalyptus maculata* (N-donor) and *Casuarina cunninghamiana* (N-receiver) (B) after 12 months growth in the glasshouse. Both the N-donor and the N-receiver were fed continuously with external ^{14}N from transplanting. ^{15}N was supplied to the N-donor only 4 weeks before harvesting; the N-receiver was deprived of external N for the same period [Values are Means, n = 9, different letter (a, b) signifies difference at P = 0.05].

Treatments	N-transfer								
	% $N_{transfer}$			$N_{transfer}$ (mg/plant)			% NDFT		
	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total
ExperimentA: N-transfer from *Casuarina (N-donor) to *Eucalyptus* (N-receiver)									
**C5→E5*	13.25a	14.50a	14.00a	79.41a	36.82a	116.22a	51.05a	39.28a	45.16a
**C4→E4*	5.15b	11.37a	11.03a	14.10b	13.01b	27.11b	17.18b	24.49b	20.84b
**C3→E3*	1.74c	4.88b	4.55b	7.04c	5.04c	12.08c	11.37c	13.57c	12.47c
**C2→E2*	0.88c	1.42b	1.37b	2.84c	1.32c	4.15c	7.56c	5.80c	6.68c
**C1→E1*	—	—	—	—	—	—	—	—	—
***C5→E5*	10.41a	11.83a	11.12a	50.09a	16.68a	66.77a	35.10a	22.33a	28.72a
***C4→E4*	7.21b	5.60b	6.41b	19.29b	6.68b	25.97b	20.37b	10.26b	15.32b
***C3→E3*	1.89c	3.05c	2.47c	7.92c	2.56c	10.47c	10.60c	5.61c	8.11c
***C2→E2*	0.97c	2.55c	1.76c	3.46c	1.81c	5.27c	5.79c	4.84c	5.32c
***C1→E1*	—	—	—	—	—	—	—	—	—
ExperimentB: N-transfer from *Eucalyptus* (N-donor) to *Casuarina* (N-receiver)									
**E5→C5*	51.04a	49.82a	50.11a	195.44a	73.19a	268.63a	23.99a	56.11a	40.05a
**E4→C4*	20.03b	33.76b	31.16b	39.40b	29.90b	69.30b	6.02b	33.64b	19.83b
**E3→C3*	15.69b	31.28b	29.15b	9.89c	14.11c	24.00c	3.55c	15.14c	9.35c
**E2→C2*	6.06c	10.85c	10.12c	2.33c	3.00c	5.34c	1.62c	5.26c	3.44c
**E1→C1*	—	—	—	—	—	—	—	—	—
***E5→C5*	54.85a	33.45a	44.15a	242.40a	36.53a	278.93a	30.42a	31.18a	30.80a
***E4→C4*	34.24b	17.48b	25.86b	91.62b	12.58b	104.20b	14.66b	13.34b	13.90b
***E3→C3*	14.27c	8.55c	11.41c	11.87c	3.55c	15.42c	3.93c	3.67c	3.80c
***E2→C2*	5.32c	5.27c	5.30c	3.61c	1.43c	5.03c	1.61c	1.82c	1.72c
***E1→C1*	—	—	—	—	—	—	—	—	—

*labeled with $^{15}NH_4NO_3$, *labeled with $NH_4{}^{15}NO_3$.

3.4 ^{15}N Labeling Studies Show that More N Moves to *Casuarina*

The data presented in Table 3.6 were the average of pairs 2 and 3, or 4 and 5 for the four Experiments I, II, III and IV, respectively. Significantly higher bidirectional and net N-transfer were also found between the dual nodulated/mycorrhizal and the sole mycorrhizal pairs, and between the 6-months- and 12-months-old pairs (Table 3.7).

The results showed that N-transfer between *Casuarina* and *Eucalyptus* occurred bidirectionally in both 6-months- and 12-months-old seedlings with all four different forms of ^{15}N label supplied (Table 3.6). Surprisingly, there was a 2 – 4 times higher net gain in N by the N_2-fixing *Casuarina*, but not by the non-N_2-fixing *Eucalyptus*, in both 6-months- and 12-months-old plants, with only one exception (Table 3.7). Meanwhile, N-transfer to *Casuarina* was similar to that to *Eucalyptus* in the sole mycorrhizal pairs, but the % and amount of N-transfer, and the % of N in the receiver derived from the transfer (%NDFT), were generally significantly higher in the nodulated/mycorrhizal pairs than in the non-nodulated/mycorrhizal pairs. In general, the 12-months-old pairs had a much higher % N-transfer and higher amount of N-transferred than the 6-months-old pairs. However, the %NDFT was on the same scale regardless of N-transfer direction or age of the plants.

Table 3.7. Bidirectional and net nitrogen transfer in *Casuarina/Eucalyptus* pairs♦

	Bidirectional transfer (mg)		Net transfer (mg)	
Treatments	Sole myc-pairs♦	Dual fr/m pairs	Sole myc-pairs	Dual fr/m pairs
N-source	$*C_m \rightarrow E_{(nm)m}$ + $*E_m \rightarrow C_{(nm)m}$	$*C_{fr/m} \rightarrow E_m$ + $*E_m \rightarrow C_{fr/m}$	$*C_m \rightarrow E_{(nm)m}$ − $*E_m \rightarrow C_{(nm)m}$	$*C_{fr/m} \rightarrow E_m$ − $*E_m \rightarrow C_{fr/m}$
6-months-old seedlings				
$(^{15}NH_4)_2SO_4$	1.9*(B, B)*	24.1*(B, A)*	+0.5*(B, B)*	−14.2*(C, A)*
$K^{15}NO_3$	2.0*(B, B)*	21.2*(B, A)*	−0.1*(B, B)*	− 6.6*(C, A)*
12-months-old seedlings				
$^{15}NH_4NO_3$	19.4*(A, B)*	241.2*(A, A)*	−3.4*(A, B)*	−96.8*(B, A)*
$NH_4{}^{15}NO_3$	18.1*(A, B)*	237.9*(A, A)*	−2.4*(A, B)*	−145.2*(A, A)*

♦Abbreviations also see Table 3.6; *labeled with 4.0mM 98.0 atom % ^{15}N for 4 weeks before harvesting while withholding N-supplementation to its partner concurrently.

Table 3.6. Nitrogen transfer in *Casuarina/Eucalyptus or* soybean/*Sorghum* pairs

Treatments	N-transfer (%)				N-transferred (mg/plant)				NDFT (%)			
	Sole myc-pairs		Dual fr/m pairs		Sole myc-pairs		Dual fr/m pairs		Sole myc-pairs		Dual fr/m pairs	
N-source	$*C_m \rightarrow E_{(nm)m}$	$*E_m \rightarrow C_{(nm)m}$	$*C_{fr/m} \rightarrow E_m$	$*E_m \rightarrow C_{fr/m}$	$*C_m \rightarrow E_{(nm)m}$	$*E_m \rightarrow C_{(nm)m}$	$*C_{fr/m} \rightarrow E_m$	$*E_m \rightarrow C_{fr/m}$	$*C_m \rightarrow E_{(nm)m}$	$*E_m \rightarrow C_{(nm)m}$	$*C_{fr/m} \rightarrow E_m$	$*E_m \rightarrow C_{fr/m}$
6-months-old seedlings (5 months nutrition with 4.0mM ^{14}N)												
$(^{15}NH_4)_2SO_4$	0.9*(B, B)*	1.5*(B, B)*	2.3*(B, B)*	20.5*(B, A)*	1.2*(B, C)*	0.7*(B, C)*	5.0*(C, B)*	19.2*(B, A)*	2.0*(B, B)*	0.8*(B, B)*	5.0*(B, A)*	5.3*(B, A)*
$K^{15}NO_3$	1.2*(B, B)*	2.9*(B, B)*	2.7*(B, B)*	12.2*(B, A)*	1.0*(B, C)*	1.0*(B, C)*	7.3*(C, B)*	13.9*(B, A)*	2.1*(B, B)*	1.6*(B, B)*	7.6*(B, A)*	6.3*(B, A)*
12-months-old seedlings (11 months nutrition with 4.0mM ^{14}N)												
$^{15}NH_4NO_3$	2.9*(A, C)*	14.4*(A, B)*	12.8*(A, B)*	40.6*(A, A)*	8.0*(A, C)*	11.4*(A, C)*	72.2*(A, B)*	169.0*(A, A)*	9.6*(A, B)*	4.7*(A, B)*	33.0*(A, A)*	30.0*(A, A)*
$NH_4{}^{15}NO_3$	2.1*(A, C)*	8.6*(A, B)*	8.8*(A, B)*	35.0*(A, A)*	7.9*(A, C)*	10.2*(A, C)*	46.4*(B, B)*	191.6*(A, A)*	6.7*(A, B)*	3.8*(A, B)*	22.0*(A, A)*	22.4*(A, A)*

*labeled with 4.0mM 98.0 atom % ^{15}N for 4 weeks before harvesting while withholding N-supplementation to its partner concurrently. C: *Casuarina cunninghamiana*; E: *Eucalyptus maculata*; m or myc: mycorrhizal infected; (nm)m: initially non-mycorrhizal but mycorrhizal colonised through its partner during experiment; fr: *Frankia* nodulated. NDFT: N in the receiver derived from transfer. Values are means, n = 18, different letter (*A, A*) signifies difference at P = 0.01 for the individual parameter in the same column and row, respectively.

CHAPTER 4 NITROGEN TRANSFER BETWEEN *CASUARINA* AND *EUCALYPTUS*, OR SOYBEAN AND *SORGHUM*, THROUGH COMMON MYCORRHIZAL NETWORKS: ^{15}N NATURAL ABUNDANCE STUDIES

4.1 Introduction

When measured against atmospheric N_2 as a reference, the naturally occurring isotope ratio of $^{15}N/^{14}N$, is termed $\delta^{15}N$. $\delta^{15}N$ and ^{15}N enrichment are quite different because $\delta^{15}N$ values are much smaller than those of ^{15}N-enriched materials. ^{15}N comprises only 0.3663% of total air N_2, but can be greater than 99.5% in ^{15}N-enriched chemicals. $\delta^{15}N$ has been used in numerous investigations including studies of N_2-fixation (Shearer and Kohl, 1986; Virginia *et al.*, 1989; Knowles and Blackburn, 1993; Unkovich *et al.*, 1994, 2001; Nelsen and Orcutt, 1996; Boddey *et al.*, 2000; Tjepkema *et al.*, 2000), N source utilisation and N cycling processes in terrestrial ecosystems (Handley and Raven, 1992; Huss-Danell, 1997; Handley and Scrimgeour, 1997; Högberg 1997; Evans, 2001; Robinson, 2001). For example, it has been used to estimate N_2-fixation of soybean (George *et al.*, 1993; Song *et al.*, 1995) and *Casuarina* (Mariotti *et al.*, 1992), N balances of sorghum in intercropping systems (Tobita *et al.*, 1994; Adu *et al.*, 1997; Kamayama *et al.*, 1999), and in *Eucalyptus* plantations (Jonsson *et al.*, 1996), and effects of AM fungi on $\delta^{15}N$ variation of casuarinas (Wheeler *et al.* 2000). Wheeler *et al.* (2000) also indicated that there was a significant linear relationship between the natural logarithms of cladode N and $\delta^{15}N$, when four *Casuarina* species were inoculated with *Frankia* alone or together with an AM fungus. In general, these studies indicated that the $\delta^{15}N$ method was reliable for assessing N utilisation and has a comparative advantage over the conventional N difference and ^{15}N enrichment methods for estimation of N_2-fixation, especially in cases where no suitable non-N_2-fixing reference plant was available.

Högberg and Johannisson (1993) indicated that N loss was strongly correlated with $\delta^{15}N$ values of forest soils, where the lighter ^{14}N tended to be lost from the system more easily than the heavier ^{15}N. Studies suggested that ECM associations could potentially contribute to $\delta^{15}N$ variations of the host plants (Michelsen *et al.*, 1996;

Högberg, 1997; Handley and Scrimgeour, 1997; Hobbie *et al.*, 1999a, b; 2000). $\delta^{15}N$ values were also higher in AM than in non-AM *Ricinus communis*, *Acacia* and *Sorghum* (Handley *et al.*, 1993; Azcon-G.-Aguilar *et al.*, 1998; Fonseca *et al.*, 2001). However, Michelsen and Sprent (1994) reported that, although N_2-fixation and shoot biomass were increased for four AM infected, nodulated *Acacia* species, $\delta^{15}N$ values were not related to their shoot N contents. There was no significant effect of AM on $\delta^{15}N$ values of non-nodulated *Acacia nilotica* or *E. globulus,* neither. The reason for this probably was that ECM fungi might have the capacity to access soil organic N in addition to inorganic N (Högberg, 1990; Michelsen and Sprent, 1994). A general $\delta^{15}N$ value pattern was found: non-mycorrhizal/AM > ECM ≥ ericoid mycorrhizal plants (Michelsen *et al.*, 1998). Fractionation during mycorrhizal transfer of N provided the best explanation for similar patterns observed in the field (Hobbie *et al.*, 2000). Thus, the question is whether ^{15}N natural abundance can be employed to investigate N-transfer between ECM or AM plants. Nevertheless, it is surprising that $\delta^{15}N$ has not been more widely used, or at least as much as enriched ^{15}N in N-transfer studies. In deed, there are several other reasons why the ^{15}N natural abundance has not been more widely used. These range from the heterogeneity of isotope signatures in soils (Hansen *et al.*, 1987; Pate *et al.*, 1993), the nonidentical $\delta^{15}N$ values in all parts of one plant (Shearer and Kohl, 1986), the difficulties in selection of an appropriate reference plant and to the high cost of mass spectrometers of sufficient sensitivity (Knowles and Blackburn, 1993; Griffiths, 1998; Peoples and Herridge, 1999; Boddey *et al.*, 2000; Unkovich and Pate, 2000, 2001; Unkovich *et al.*, 1994, 2001).

Binkley *et al.* (1985) were the first to use $\delta^{15}N$ for tracing N transfer between non-mycorrhizal red alder and Douglas-fir trees. They suggested that fractionations of N isotopes in the soil make this difficult. $\delta^{15}N$ values of the red alder leaves and the mineral and total soil-N pools in the mixed stand were compared from the following four sites: (1) a pure stand of Douglas-fir; (2) a stand of mixed conifers; (3) a stand of Douglas-fir mixed with N_2-fixing Sitka alder, and (4) a stand of conifers mixed with the N_2-fixing red alder. The results indicated that there was no consistent pattern, although $\delta^{15}N$ of the alders and non-N_2-fixing trees differed significantly among the four stands. They concluded that the ^{15}N natural abundance method did

not provide a simple means of evaluating N-transfer, even though isotope discrimination clearly occurred during N cycling at these forest sites at the ecosystem level. Kohls *et al.* (1994) found there was no isotopic change in non-N_2-fixing plants growing within 1 m of non-nodulated actinorhizal N_2-fixing *Dryas*. However, the leaf $\delta^{15}N$ value was closer to that of atmospheric N_2 in non-N_2-fixing plants growing within 1 m of nodulated *Dryas* that showed an active N_2-fixation ability. The non-N_2-fixing plants also had significantly higher foliar N contents. These results indicated that some symbiotically fixed N had been translocated from the N_2-fixing plants to the non-N_2-fixing ones. N_2-fixing plants, therefore, could stimulate the performance of adjacent non-N_2-fixing plants that had already established, through transfer of fixed-N. van Kessel *et al.* (1994) suggested that a decline over time in $\delta^{15}N$ of understory plants, viz. beneath N_2-fixing *Leucaena leucocephala* shrubs was evidence of a transfer of fixed N. The *L. leucocephala* obtained about 75% of its N supply from N_2-fixation during the first 2 years of growth. The mean $\delta^{15}N$ values of non-N_2-fixing understory plants dropped from 7.3 ‰ to 3.4 ‰, 0.7 ‰ and 0.9 ‰ after 1, 2, 4 and 7 years of growth, respectively. The understory vegetation and *L. leucocephala* showed a similar downward trend in $\delta^{15}N$ with time and had essentially the same average $\delta^{15}N$ after 7 years. The authors claimed this was the first direct evidence for the possibility of using the ^{15}N natural abundance technique to investigate N movement between an N_2-fixing tree and its non-N_2-fixing understorey vegetation within an agro-ecosystem. Unfortunately, the root mycorrhization status for either the N_2-fixing or the non-N_2-fixing plant was not evaluated in these three studies. Therefore, whether mycorrhizal colonisation had an effect on the $\delta^{15}N$ variations in all the stands where they were sampled was not clear.

So far, there is no conclusive evidence that the $\delta^{15}N$ natural abundance technique can be used to investigate either one-way or two-way N-transfer between N_2-fixing and non-N_2-fixing plants. Furthermore, neither do we know if any N-transfer occurs between plants of the same species (either non-N_2-fixing or N_2-fixing plant) in pure system. In the following glasshouse experiments, with or without ECM hyphal interconnection, the $\delta^{15}N$ technique was used to trace N-transfer amongst *Eucalyptus* or *Casuarina* in pure or mixed stands. N-transfer between soybean and *Sorghum*, with or without common AM mycorrhizal networks, was also investigated.

4.2 N-Transfer in Pure Systems of Non-N_2-fixing or N_2-fixing Plants

4.2.1 N-transfer in pure system of *Eucalyptus* pairs

4.2.1.1 *Experimental Design*

Table 4.1 shows the experimental design for N-transfer in pure system of *Eucalyptus*.

4.2.1.2 *Results*

4.2.1.2.1 *Formation of common mycorrhizal networks*

The non-mycorrhizal *EA1* and *EB1* remained largely uncolonised. In contrast, the originally non-mycorrhizal *EB2* became infected through its mycorrhizal partner *EA2*. The plants in *EA2*, *EA3* and *EB3*, aseptically cultured with *Pisolithus tinctorius*, maintained and developed further their mycorrhization by the end of the experiment (Figure 4.1). Moreover, the total mycorrhizal infection was much greater in the mycorrhizal than in the non-mycorrhizal roots. Together with the observation of mycorrhization by the light and the Environmental Scanning Electron Microscopes, the results showed that (1) the aseptically synthesised mycorrhizal plants developed further their mycorrhization in the open environment, and (2) a common underground ectomycorrhizal link had been successfully established between the mycorrhizal and the initially non-mycorrhizal *Eucalyptus* roots.

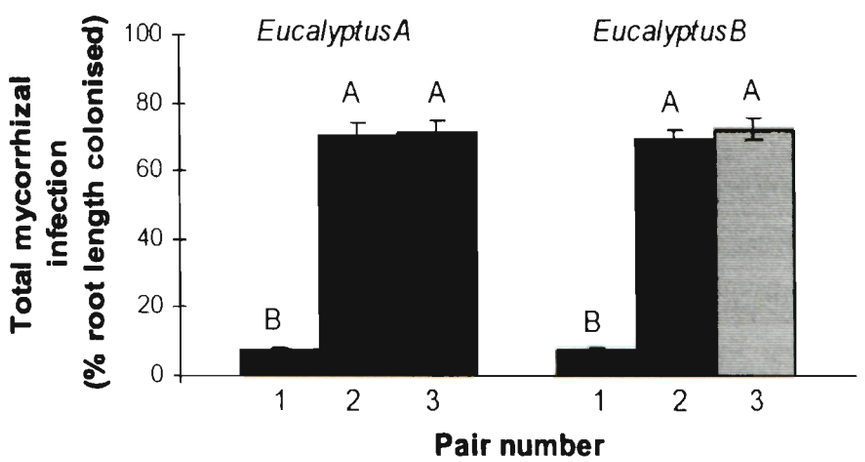

Figure 4.1. Mycorrhizal root colonisation of *EucalyptusA* (N-donor) and *EucalyptusB* (N-receiver). [Means ± SE, n = 9; different letter (A, B) signifies difference at P = 0.01].

Table 4.1. Pairing of 12-months-old *Eucalyptus maculataA* (N-donor) and *E. maculataB* (N-receiver) to identify N-transfer between plants. Both N-donor and N-receiver were continuously fed with external ^{14}N from the time of transplanting, but the N-receiver was deprived of N for 4 weeks before harvesting.

	N-donor		N-receiver	Code
Pair 1:	$EucalyptusA_{control}$	+	$EucalyptusB_{control}$	EA1→EB1
Pair 2:	$EucalyptusA_{ectomycorrhiza}$	+	$EucalyptusB_{-ectomycorrhiza}$	EA2→EB2
Pair 3:	$EucalyptusA_{ectomycorrhiza}$	+	$EucalyptusB_{ectomycorrhiza}$	EA3→EB3

4.2.1.2.2 *Responsiveness of Eucalyptus to mycorrhization*

Shoot, root or total (shoot plus root) biomass showed a positive response to mycorrhization (Figure 4.2). For both *EucalyptusA* and *EucalyptusB*, the shoot, root and total biomass differed significantly between the non-mycorrhizal and the mycorrhizal plants (original data not shown). No significant differences in dry matter were found in *EucalyptusA* and *EucalyptusB* pairs. The result indicated that growth in *Eucalyptus* could be greatly enhanced by mycorrhization.

Nitrogen content was also positively and highly significantly related to mycorrhizal infection, but no difference was observed between *EucalyptusA* and *EucalyptusB* (Figure 4.3). The result revealed that the mycorrhizal fungus *P. tinctorius* had a positive influence on N acquisition and accumulation in *Eucalyptus* plants.

A positive correlation was found between biomass and N content in *Eucalyptus* (Figure 4.4), suggesting that biomass responded linearly to N increase in *Eucalyptus*.

4.2.1.2.3 *Relationship between N concentration or content and $\delta^{15}N$ value*

For all three measurements, shoot, root and total, $\delta^{15}N$ values decreased with increasing N concentration (Figures 4.5a, b and c) or N content (Figures 4.5d, e and f) in both *EucalyptusA* and *EucalyptusB*. In addition, all three parameters of shoot, root and total $\delta^{15}N$ values were significantly different between the mycorrhizal and the non-mycorrhizal eucalypts.

4.2.1.2.4 *Nitrogen transfer between Eucalyptus plants*

Table 4.2 indicates that N was translocated between mycorrhizal *Eucalyptus* plants but no significant difference in % N-transfer, the amount of N-transfer and % NDFT was found between pairs 2 and 3.

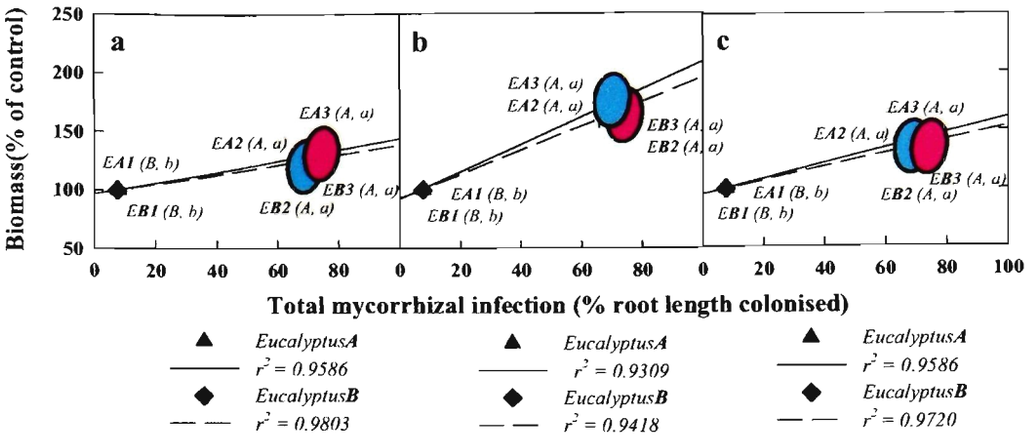

Figure 4.2. Relationships between root total mycorrhizal colonisation and shoot (a), root (b) and total (shoot plus root) (c) biomass production of *EucalyptusA* (N-donor) and *EucalyptusB* (N-receiver). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

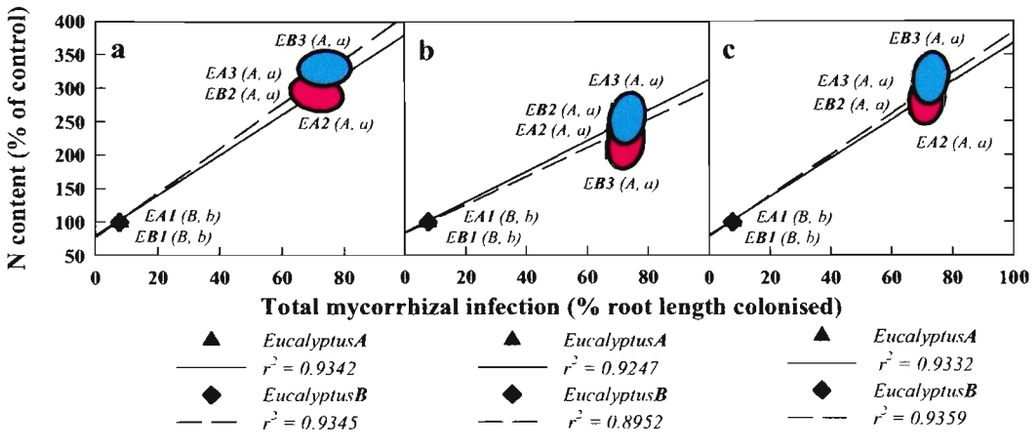

Figure 4.3. Relationships between root total mycorrhizal colonisation and shoot (a), root (b) and total (shoot plus root) (c) N content of *EucalyptusA* (N-donor) and *EucalyptusB* (N-receiver). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

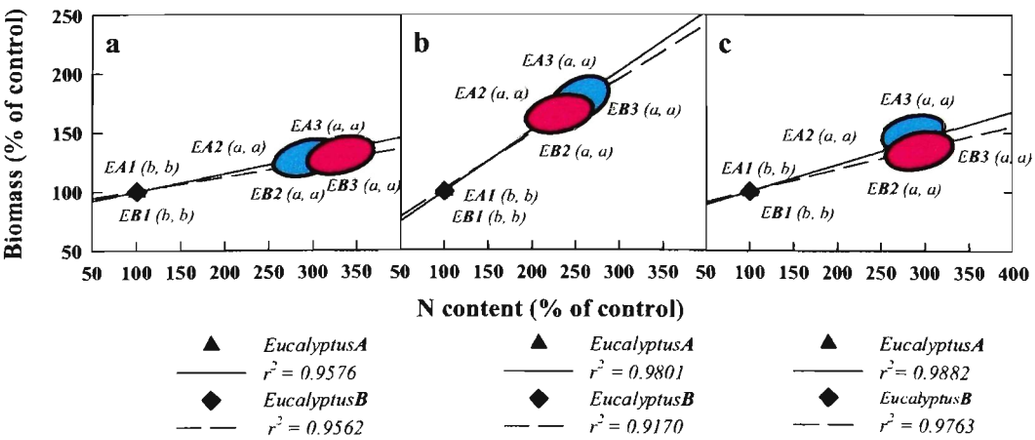

Figure 4.4. Relationships between N content and biomass production in shoot (a), root (b) and total (shoot plus root) (c) of *EucalyptusA* (N-donor) and *EucalyptusB* (N-receiver). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

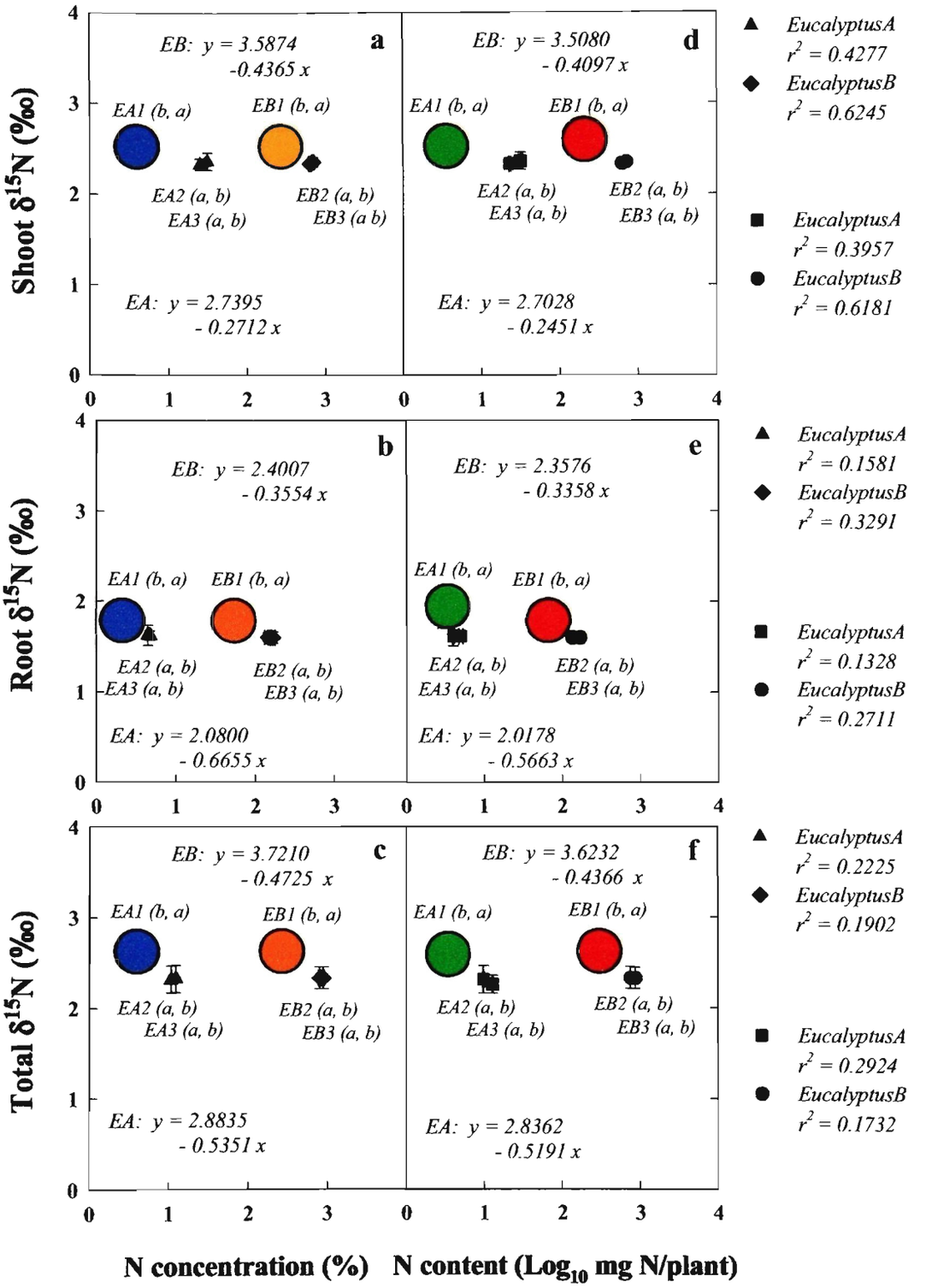

Figure 4.5. Relationships between N concentration (a-c) or N content (d-f) and δ^{15}N value of *EucalyptusA* (N-donor) and *EucalyptusB* (N-receiver). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

Table 4.2. N-transfer between 12-months-old *E. maculataA* (N-donor) and *E. maculataB* (N-receiver). Both N-donor and N-receiver were continuously fed with external ^{14}N from the time of transplanting but the N-receiver was deprived of N for 4 weeks before harvesting [Values are Means, n = 9, different letter (a, b) signifies difference at P = 0.05].

Treatments	N-transfer											
	$\delta^{15}N$ (‰)▲			% $N_{transfer}$			$N_{transfer}$ (mg/plant)			% NDFT		
	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total
EA3→EB3	2.35b	1.60b	2.33b	8.44a	11.16a	9.80a	59.82a	18.48a	78.30a	8.49a	11.30a	9.90a
EA2→EB2	2.34b	1.60b	2.33b	9.07a	10.93a	10.00a	59.14a	16.10a	75.24a	9.47a	12.05a	10.76a
EA1→EB1	2.57a	1.80a	2.61a	—	—	—	—	—	—	—	—	—

▲$\delta^{15}N$ of N-receiver.

4.2.2 N-transfer in pure system of *Casuarina* pairs

4.2.2.1 *Experimental Design*

Table 4.3 shows the experimental design for N-transfer in pure system of casuarinas.

Table 4.3. Pairing of 12-months-old *Casuarina cunninghamianaA* (N-donor) and *C. cunninghamianaB* (N-receiver), and *C. cunninghamianaX* (N-donor) and *C. cunninghamianaY* (N-receiver) to identify N-transfer between plants. Both N-donor and N-receiver were continuously fed with external ^{14}N from the time of transplanting but the N-receiver was deprived of N for 4 weeks before harvesting.

N-donor		N-receiver	Code
A. N-transfer from *CasuarinaA to CasuarinaB*			
Pair 1:	$CasuarinaA_{control}$	+ $CasuarinaB_{control}$	CA1→CB1
Pair 2:	$CasuarinaA_{Frankia}$	+ $CasuarinaB_{-ectomycorrhiza}$	CA2→CB2
Pair 3:	$CasuarinaA_{ectomycorrhiza}$	+ $CasuarinaB_{-ectomycorrhiza}$	CA3→CB3
Pair 4:	$CasuarinaA_{Frankia}$	+ $CasuarinaB_{ectomycorrhiza}$	CA4→CB4
Pair 5:	$CasuarinaA_{Frankia + ectomycorrhiza}$	+ $CasuarinaB_{Frankia}$	CA5→CB5
B. N-transfer from *CasuarinaX to CasuarinaY*			
Pair 1:	$CasuarinaX_{control}$	+ $CasuarinaY_{control}$	CX1→CY1
Pair 2:	$CasuarinaX_{-ectomycorrhiza}$	+ $CasuarinaY_{Frankia}$	CX2→CY2
Pair 3:	$CasuarinaX_{-ectomycorrhiza}$	+ $CasuarinaY_{ectomycorrhiza}$	CX3→CY3
Pair 4:	$CasuarinaX_{ectomycorrhiza}$	+ $CasuarinaY_{Frankia}$	CX4→CY4
Pair 5:	$CasuarinaX_{Frankia}$	+ $CasuarinaY_{Frankia+ectomycorrhiza}$	CX5→CY5

4.2.2.2 *Results*

4.2.2.2.1 *Formation of common mycorrhizal networks*

Plants in pair 1 and pair 2 kept their uninfected status, and those incubated with *Pisolithus tinctorius* aseptically in pairs 3, 4 and 5 maintained their mycorrhization in the glasshouse (Figure 4.6). The non-mycorrhizal plants in pairs 3, 4 and 5 became colonised by growing with the mycorrhizal partners at the end of the experiment. The root mycorrhizal colonisation was thus much greater in the mycorrhizal than in the non-mycorrhizal plants. Together with the observation of mycorrhization by the light and the Environmental Scanning Electron Microscopes, the results showed that (1) the aseptically synthesised ectomycorrhizal plants developed further their colonisation in the open environment, and (2) a common underground ectomycorrhizal link was successfully set up between the mycorrhizal and the initially non-mycorrhizal *Casuarina* roots.

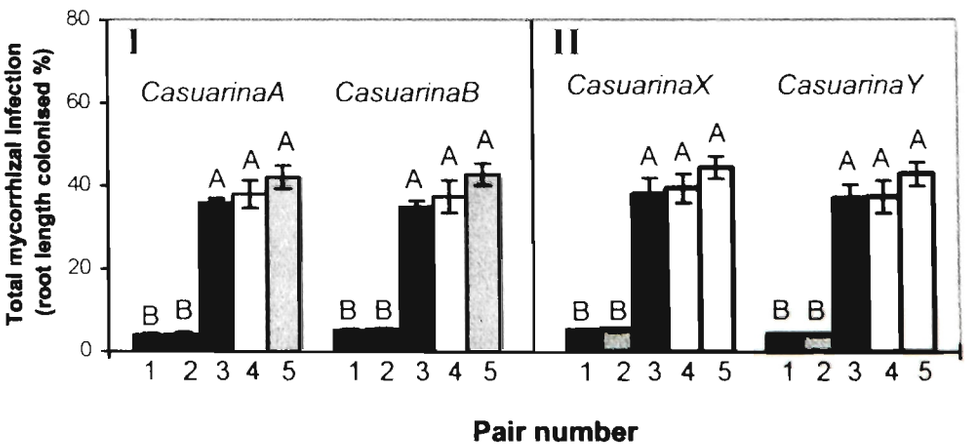

Figure 4.6. Mycorrhizal root colonisation of *CasuarinaA* (N-donor) and *CasuarinaB* (N-receiver) (I), and *CasuarinaX* (N-donor) and *CasuarinaY* (N-receiver) (II). [Means ± SE, n = 9, different letter (A, B) signifies difference at P = 0.01].

4.2.2.2.2 *Biological N_2-fixation in casuarinas*

Table 4.4. $\delta^{15}N$ values, percentage of biological nitrogen fixation (%N_{BNF}) and specific nodule activity (mg N fixed/mg nodule dry weight) in 12-months-old *Casuarina cunninghamianaA* (N-donor) and *C. cunninghamianaB* (N-receiver), and *C. cunninghamianaX* (N-donor) and *C. cunninghamianaY* (N-receiver) [Means ± SE, n = 9; different letter (a, b) signifies difference at P = 0.05].

N-source	Treatment•	Age◆	$\delta^{15}N$ (‰)	% N_{BNF}	Specific nodule activity
A. N-transfer from *CasuarinaA* to *CasuarinaB*					
$(NH_4)_2SO_4$▲	*CA2(fr)*	12	-2.16a	40.99 ± 2.74b	0.0147 ± 0.0010b
$(NH_4)_2SO_4$▲	*CA4[fr/(nm)m]*	12	-2.43a	44.70 ± 1.90a	0.0172 ± 0.0008a
$(NH_4)_2SO_4$▲	*CA5(fr/m)*	12	-2.59a	46.95 ± 1.65a	0.0174 ± 0.0002a
$(NH_4)_2SO_4$▲	*CB5[fr/(nm)m]*	12	-2.46a	45.16 ± 1.29a	0.0175 ± 0.0001a
B. N-transfer from *CasuarinaX* to *CasuarinaY*					
$(NH_4)_2SO_4$▼	*CY2(fr)*	12	-2.18a	41.38 ± 1.53b	0.0148 ± 0.0008b
$(NH_4)_2SO_4$▼	*CY4[fr/(nm)m]*	12	-2.48a	45.43 ± 0.99a	0.0169 ± 0.0003a
$(NH_4)_2SO_4$▼	*CY5(fr/m)*	12	-2.57a	46.77 ± 0.24a	0.0177 ± 0.0002a
$(NH_4)_2SO_4$▼	*CX5[fr/(nm)m]*	12	-2.46a	45.21 ± 2.03a	0.0175 ± 0.0002a

•*Frankia* and/or mycorrhizal association after the experiment; ◆months; ▲fed with external ^{14}N from time of transplanting till harvest; ▼deprived of ^{14}N for 4 weeks before harvest. $\delta^{15}N$ = –6.44‰ for nodulated *Casuarina* cultivated in an N-free medium. m: mycorrhizal infected; (nm)m: initially non-mycorrhizal but mycorrhizal colonised through its partner during experiment; fr: *Frankia* nodulated; fr/(nm)m: initially non-mycorrhizal *Frankia* infected but mycorrhizal colonised through its partner during experiment.

Table 4.4 shows that both % N_{BNF} and specific nodule activity differed significantly between the nodulated mycorrhizal and the nodulated non-mycorrhizal casuarinas, no matter whether the mycorrhization was developed originally through aseptic indoor incubation or afterwards through growing with the mycorrhizal partner in the glasshouse. However, shoot $\delta^{15}N$ values were of the same order among these treatments.

4.2.2.2.3 *Responsiveness of Casuarina to mycorrhization*

Shoot, root or total biomass correlated positively with mycorrhizal colonisation (Figure 4.7). Dual *Frankia*/mycorrhizal infection had the most positive effect on the growth performance of *Casuarina*, although nodulation alone enhanced biomass production significantly more than mycorrhization alone.

Nitrogen content was also positively related to root mycorrhizal colonisation, though *CasuarinaA* or *CasuarinaY* had weaker responses with mycorrhization (r^2 = 0.2744 ~ 0.4115) than *CasuarinaB* or *CasuarinaX* (r^2 = 0.5053 ~ 0.7679) (Figure 4.8). Again, *Frankia* nodulation had a greater influence on N accumulation than mycorrhizal colonisation, but it was the N_2-fixation combining with mycorrhization that contributed the most N to *Casuarina* plants. This indicated that N_2 fixation by *Frankia* is of primary and mycorrhization of secondary importance for satisfying N requirements in *Casuarina*.

Biomass production increased linearly with increasing N content and the correlations were high in both pairs of *CasuarinaA/B* and *CasuarinaX/Y* (Figure 4.9). In addition, nodule dry matter also responded postively to increasing N content (Insets in Figure 4.9b and e). The nodulated mycorrhizal plants had the highest and the non-nodulated non-mycorrhizal plants had the lowest biomass response with N content, respectively. The nodulated non-mycorrhizal plants showed a greater response with N content than their non-nodulated mycorrhizal counterparts.

4.2.2.2.4 *Relationship between N concentration or content and $\delta^{15}N$ value*

$\delta^{15}N$ decreased with both N concentration (Figure 4.10) and N content (Figure 4.11) for all the three measurements of shoot, root and the total. A significant $\delta^{15}N$ decrease with increase in N concentration and N content was seen between the nodulated/mycorrhizal and the sole mycorrhizal plants, as it was between the latter and the controls. Nitrogen concentration was within a quite narrow range, especially in the roots, and there was no significant difference between the nodulated non-mycorrhizal and the non-nodulated mycorrhizal plants (Figure 4.10). However, N content in plants ranked as follows: the nodulated mycorrhizal > the nodulated non-mycorrhizal > the non-nodulated mycorrhizal > the non-nodulated non-mycorrhizal control plants (Figure 4.11).

4.2.2.2.5 *Nitrogen transfer between Casuarina plants*

Table 4.5 shows that N was translocated between mycorrhizal *Casuarina* plants. The % N-transfer, the amount of N-transfer and % NDFT were significantly higher in the dual *Frankia*/mycorrhizal pairs (*CA5→CB5*, *CA4→CB4*, and *CX5→CY5*, *CX4→CY4*) than in the sole mycorrhizal pairs (*CA3→CB3*, and *CX3→CY3*).

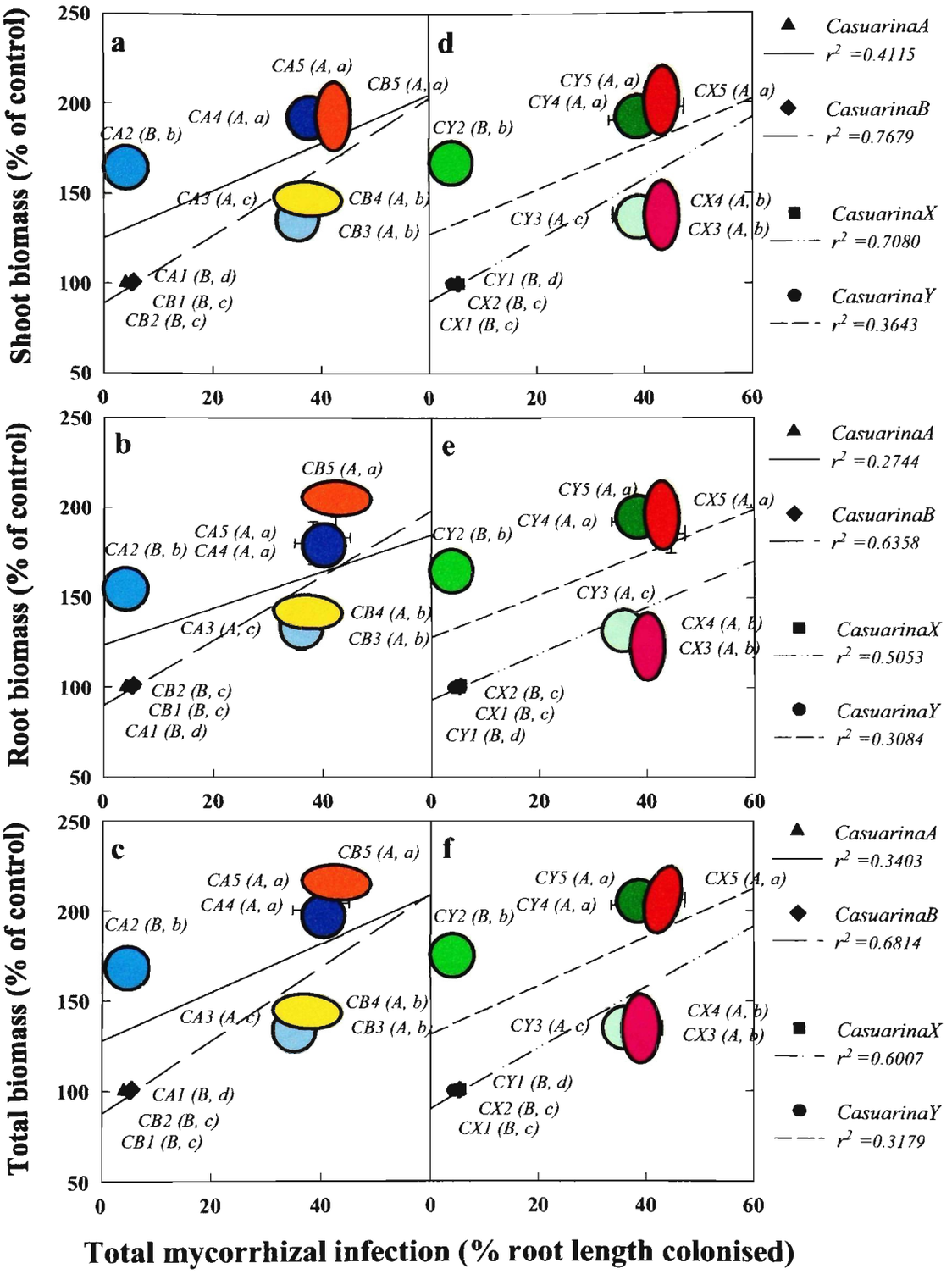

Figure 4.7. Relationships between root mycorrhizal colonisation and tissue biomass production of *CasuarinaA* (N-donor) and *CasuarinaB* (N-receiver) (a-c); and *CasuarinaX* (N-donor) and *CasuarinaY* (N-receiver) (d-f). [Means ± SE, n = 9; different letters (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively

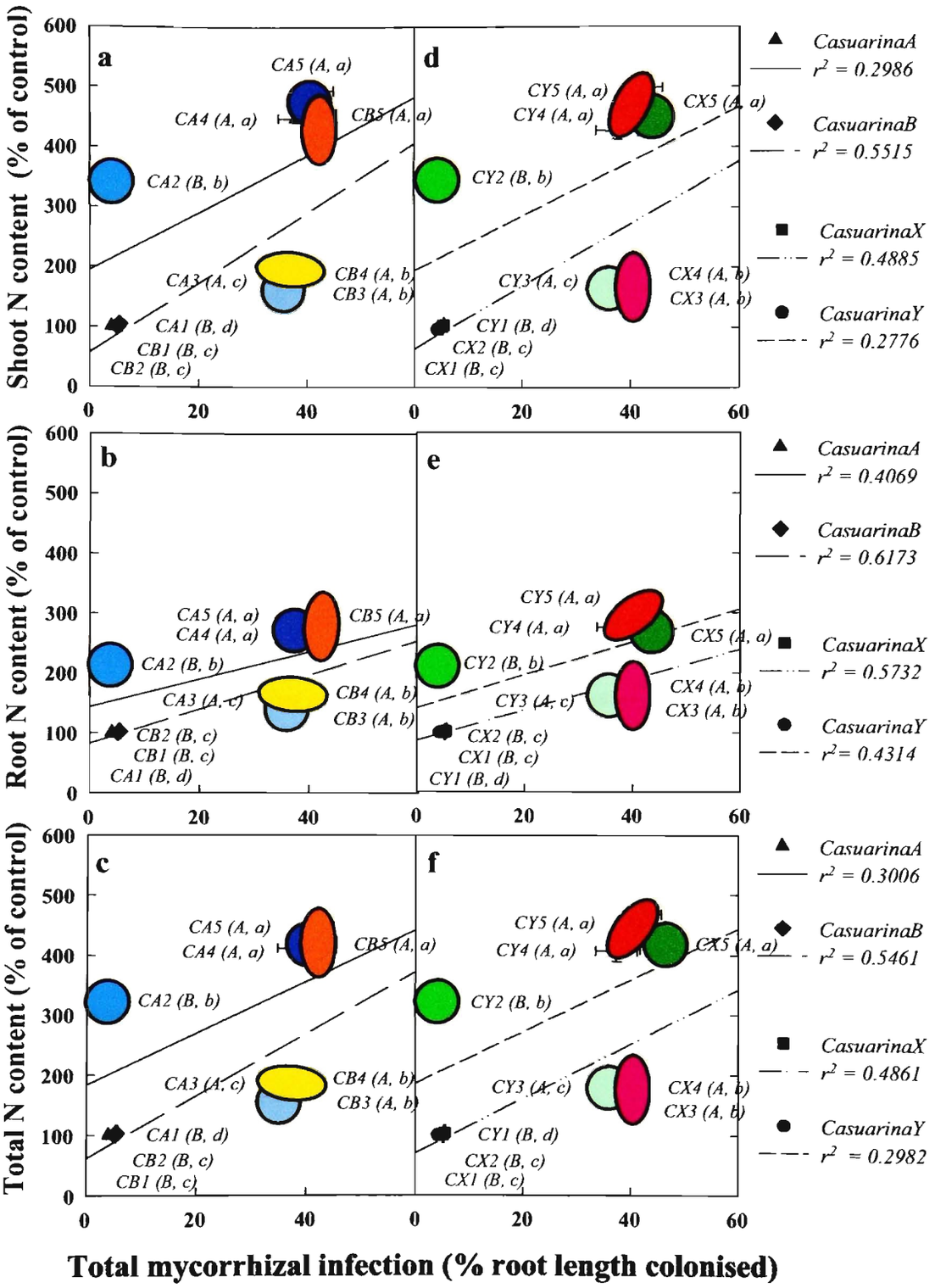

Figure 4.8. Relationships between root mycorrhizal colonisation and tissue N content production of *CasuarinaA* (N-donor) and *CasuarinaB* (N-receiver) (a-c); and *CasuarinaX* (N-donor) and *CasuarinaY* (N-receiver) (d-f). [Means ± SE, n = 9; different letters (*A*, *a*) signifies difference at P = 0.01 or 0.05 for *x*, *y* parameters, respectively

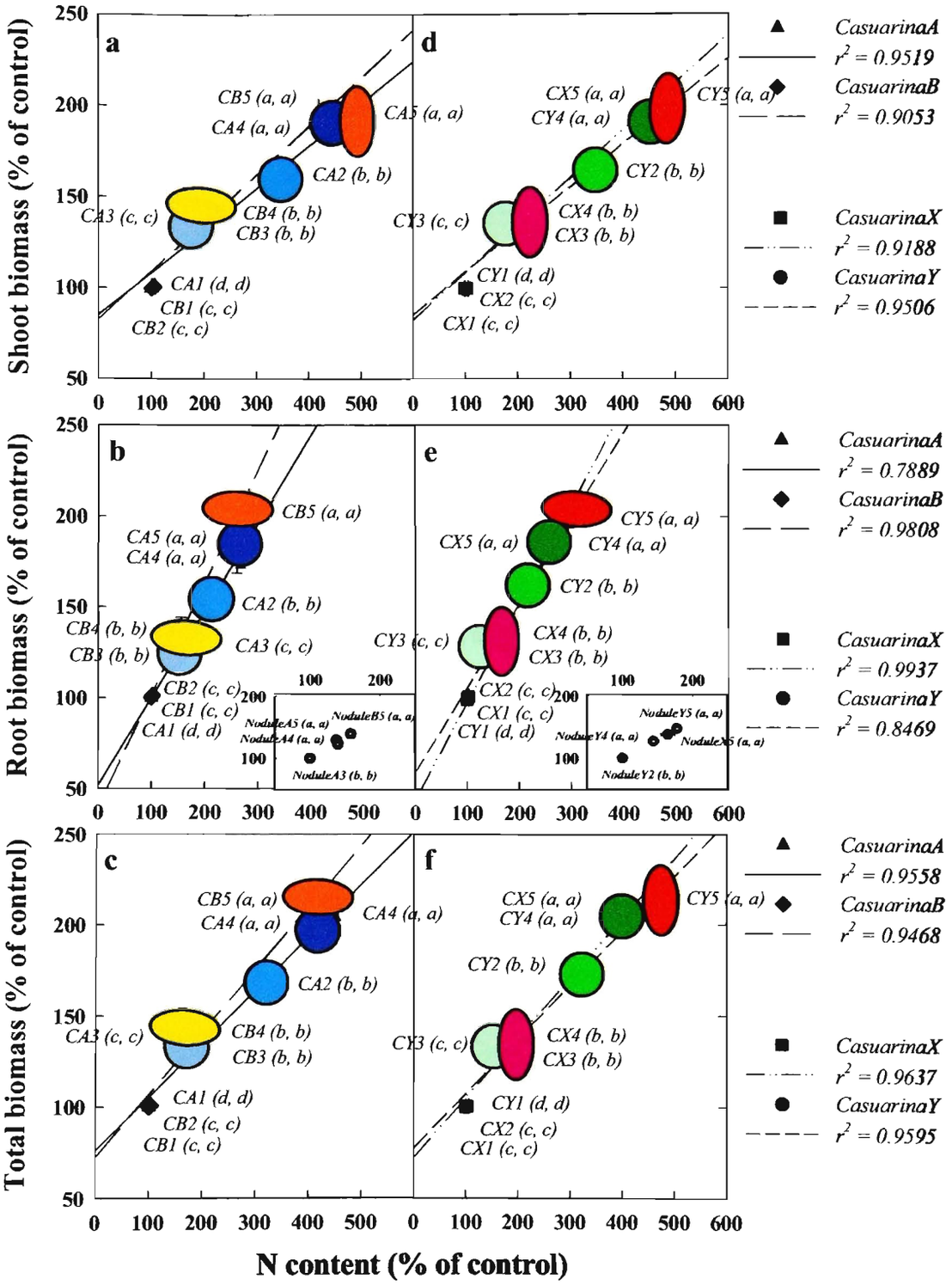

Figure 4.9. Relationships between N content and biomass production of *CasuarinaA* (N-donor) and *CasuarinaB* (N-receiver) (a-c); and *CasuarinaX* (N-donor) and *CasuarinaY* (N-receiver) (d-f). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

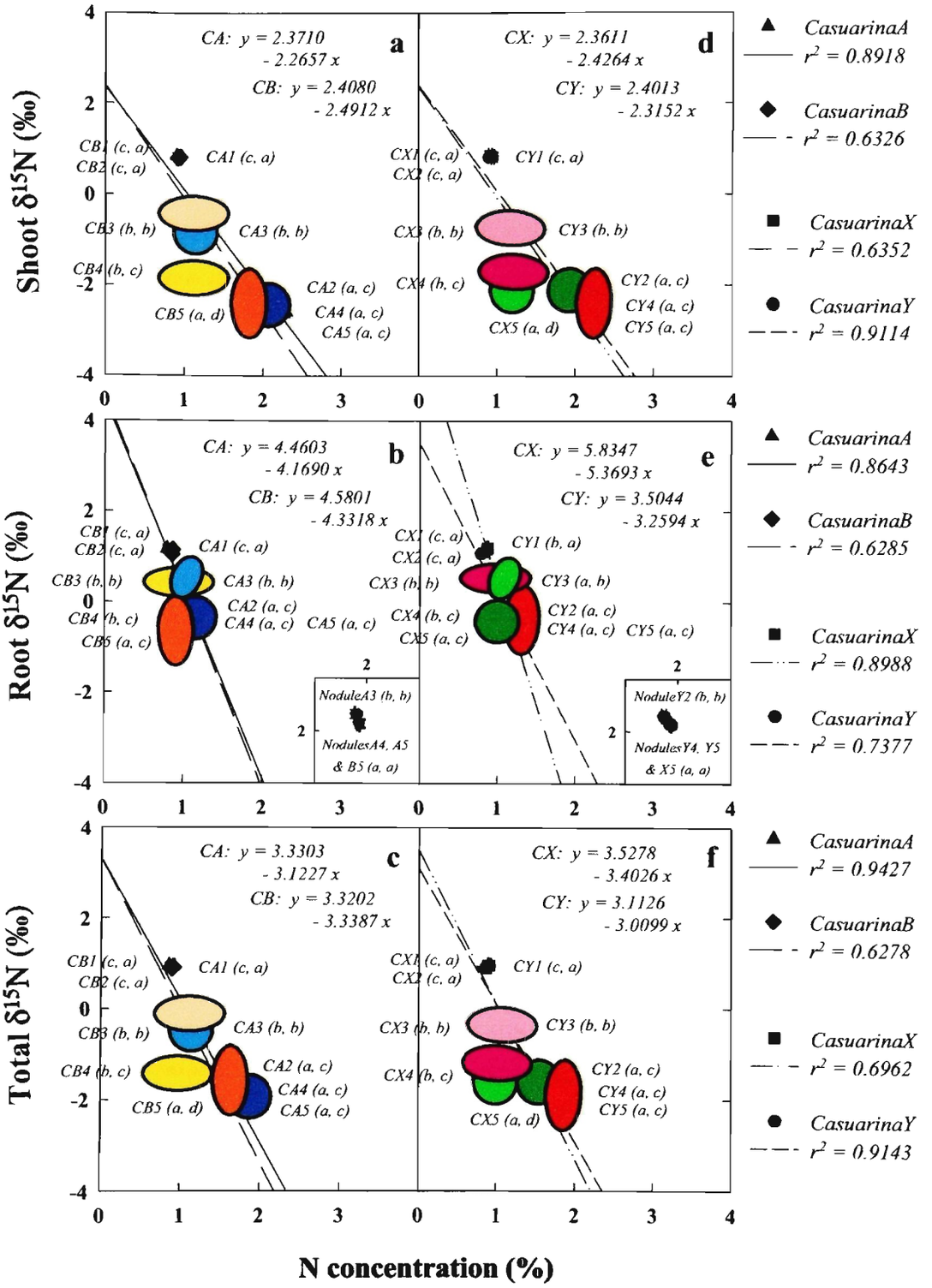

Figure 4.10. Relationships between N concentration and $\delta^{15}N$ value of *CasuarinaA* (N-donor) and *CasuarinaB* (N-receiver) (a-c); and *CasuarinaX* (N-donor) and *CasuarinaY* (N-receiver) (d-f). [Means ± SE, n = 9; different letter (*a, a*) signifies Difference at P = 0.05 for *x, y* parameters, respectively].

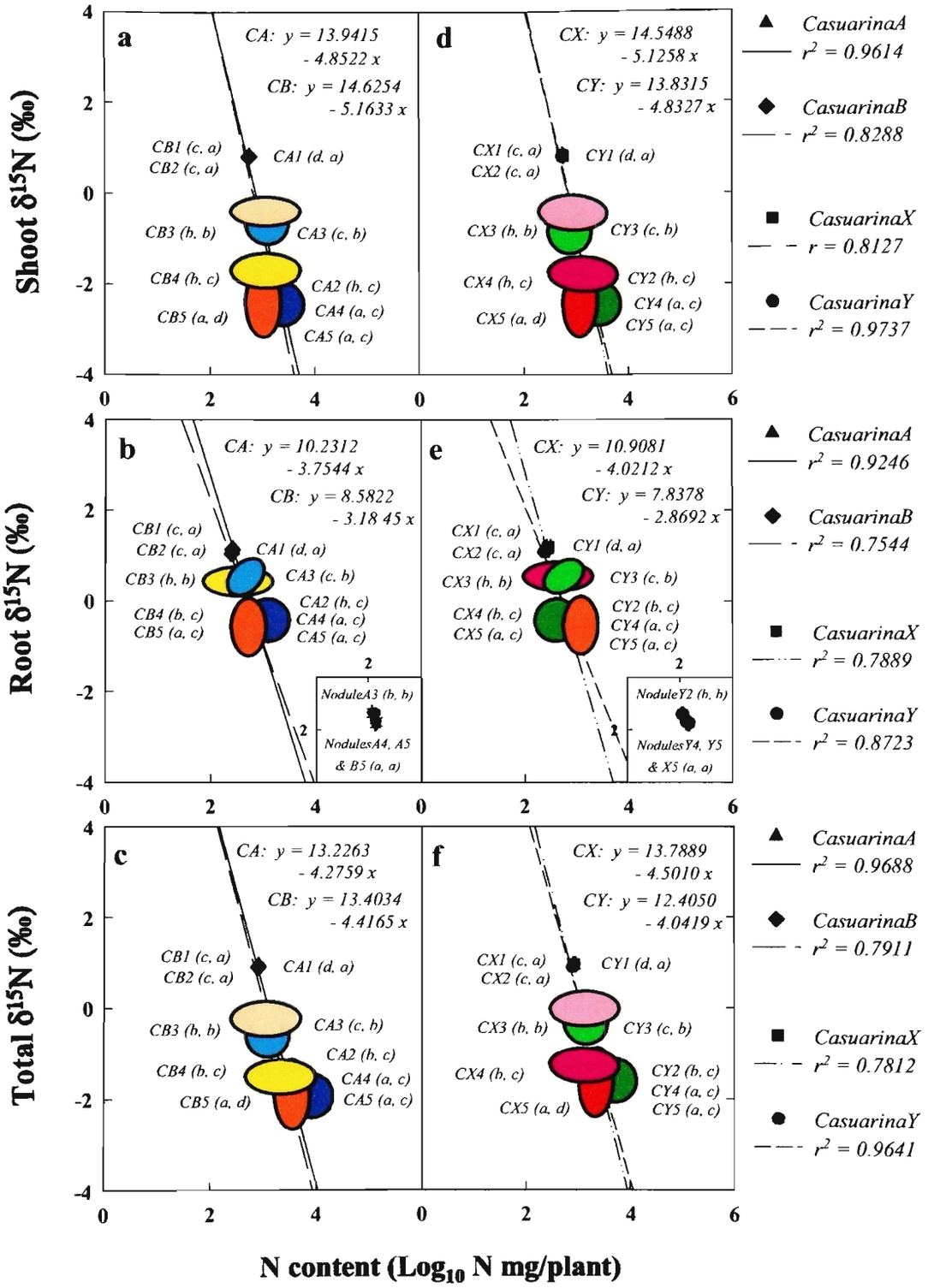

Figure 4.11. Relationships between N content and $\delta^{15}N$ value of *CasuarinaA* (N-donor) and *CasuarinaB* (N-receiver) (a-c); and *CasuarinaX* (N-donor) and *CasuarinaY* (N-receiver) (d-f). [Means ± SE, n = 9; different letter (*a, a*) signifies Difference at P = 0.05 for *x, y* parameters, respectively].

Table 4.5. N-transfer between *CasuarinaA* (N-donor) and *CasuarinaB* (N-receiver) (A), and *CasuarinaX* (N-donor) and *CasuarinaY* (N-receiver) (B) after 12-months growth in the glasshouse. Both N-donor and N-receiver were continuously fed with external ^{14}N from the time of transplanting but the N-receiver was deprived of N for 4 weeks before harvesting [Values are Means, n = 9, (a, b) signifies difference at P = 0.05].

Treatments	N-transfer											
	$\delta^{15}N$ (‰)●			% $N_{transfer}$			$N_{transfer}$ (mg/plant)			% NDFT		
	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total
A. N-transfer from *CasuarinaA to CasuarinaB*												
CA5→CB5	-2.46d	-0.25c	-1.75d	36.58a	26.81a	31.70a	976.0a	186.3a	1162.3a	39.22a	27.89a	33.56a
CA4→CB4	-1.87c	-0.18c	-1.40c	32.11a	25.80a	28.96a	319.7b	100.2b	419.9b	32.11a	25.80a	28.96a
CA3→CB3	-0.81b	0.47b	-0.45b	22.18b	14.01b	18.10b	220.9c	61.7c	282.6c	23.35b	16.55b	19.95b
CA1→CB1	0.80a	1.04a	0.87a	—	—	—	—	—	—	—	—	—
B. N-transfer from *CasuarinaX to CasuarinaY*												
CX5→CY5	-2.57c	-0.25c	-1.83c	37.54a	27.65a	32.60a	952.5a	202.3a	1154.8a	37.54a	28.58a	33.06a
CX4→CY4	-2.48c	-0.17c	-1.73c	35.81a	26.42a	31.12a	356.2b	115.7b	471.9b	35.81a	27.05a	31.43a
CX3→CY3	-0.84b	0.47b	-0.46b	22.66b	14.95b	18.81b	214.7c	63.6c	278.3c	22.66b	16.21b	19.44b
CX1→CY1	0.81a	1.08a	0.89a	—	—	—	—	—	—	—	—	—

●$\delta^{15}N$ of N-receiver; shoot and root $\delta^{15}N$ = –6.44‰ and –4.56‰ for nodulated *Casuarina* cultivated in an N-free medium, respectively.

4.3 N-transfer between Pairs of N_2-fixing and Non-N_2-fixing Plants

4.3.1 N-transfer between pairs of *Casuarina* and *Eucalyptus* (N-plus and N-minus experiments)

4.3.1.1 *Experimental Design*

Table 4.6 shows the experimental design for investigating N-transfer between *Casuarina* and *Eucalyptus* with or without external N supply.

Table 4.6. Pairing of *Casuarina cunninghamiana* and *Eucalyptus maculata* to identify N-transfer between species. ^{14}N was continuously fed to both N-donor and N-receiver from time of transplanting till harvest, except that N-receiver plants were deprived of N for 4 weeks before harvest in the N-plus experiment (12-months-old seedlings). ^{14}N was continuously fed to both N-donor and N-receiver plants for 6 weeks from time of transplanting, then withheld for another 18 weeks till harvest in the N-minus experiment (6-months-old seedlings).

N-donor		N-receiver	Code
I. N-transfer from *Casuarina to Eucalyptus*			
Pair 1: *$CasuarinaA_{control}$*	+	*$EucalyptusA_{control}$*	CA1→EA1
Pair 2: *$CasuarinaA_{ectomycorrhiza}$*	+	*$EucalyptusA_{-ectomycorrhiza}$*	CA2→EA2
Pair 3: *$CasuarinaA_{Frankia}$*	+	*$EucalyptusA_{-ectomycorrhiza}$*	CA3→EA3
Pair 4: *$CasuarinaA_{Frankia + ectomycorrhiza}$*	+	*$EucalyptusA_{-ectomycorrhiza}$*	CA4→EA4
II. N-transfer from *Eucalyptus to Casuarina*			
Pair 1: *$EucalyptusB_{control}$*	+	*$CasuarinaB_{control}$*	EB1→CB1
Pair 2: *$EucalyptusB_{-ectomycorrhiza}$*	+	*$CasuarinaB_{Frankia}$*	EB2→CB2
Pair 3: *$EucalyptusB_{ectomycorrhiza}$*	+	*$CasuarinaB_{-ectomycorrhiza}$*	EB3→CB3
Pair 4: *$EucalyptusB_{ectomycorrhiza}$*	+	*$CasuarinaB_{Frankia}$*	EB4→CB4

4.3.1.2 *Results*

4.3.1.2.1 *Formation of common ectomycorrhizal networks between Casuarina and Eucalyptus*

Both *Casuarina* and *Eucalyptus* in the non-mycorrhizal pairings maintained their non-mycorrhizal status (Figure 4.12). In contrast, all plants of *CA2*, *CA4*, *EB3* and *EB4* that were aseptically infected with *P. tinctorius* indoors developed further their mycorrhization in pots. The originally non-mycorrhizal plants of *EA2*, *EA4*, *CB3* and *CB4* became colonised to a similar extent via their mycorrhizal partners by the end of the experiment. Mycorrhization was nearly half in *Casuarina* compared to *Eucalyptus*, and was little affected by external N in both species (Figure 4.12I versus 4.12II). Along with the mycorrhizal observation by the light and the Environmental Scanning Electron Microscopes, the results demonstrated the successful formation of ECM networks between the two species regardless of external N input.

4.3.1.2.2 *Biological N_2-fixation in Casuarinas*

Table 4.7. $\delta^{15}N$ values, percentage of biological nitrogen fixation ($\%N_{BNF}$) and specific nodule activity (mg N fixed/mg nodule dry weight) in *Casuarina cunninghamianaA* and *C. cunninghamianaB* [Means ± SE, n = 9; different letter (a, b) signifies difference at P = 0.05].

N-source	Treatment•	Age◆	$\delta^{15}N$ (‰)	% N_{BNF}	Specific nodule activity
I. N-plus experiment (12-months-old seedlings)					
$(NH_4)_2SO_4$▲	*CA3(fr)*	12	-1.81b	47.15 ± 0.58b	0.0135 ± 0.0002b
$(NH_4)_2SO_4$▲	*CA4(fr/m)*	12	-2.14a	50.39 ± 1.39a	0.0172 ± 0.0004a
$(NH_4)_2SO_4$▼	*CB2(fr)*	12	-1.81b	47.48 ± 0.58b	0.0136 ± 0.0002b
$(NH_4)_2SO_4$▼	*CB4[fr/(nm)m]*	12	-2.19a	50.58 ± 0.49a	0.0181 ± 0.0003a
II. N-minus experiment (6-months-old seedlings)					
$(NH_4)_2SO_4$*	*CA3(fr)*	6	0.28a	16.36 ± 0.98b	0.0213 ± 0.0011b
$(NH_4)_2SO_4$*	*CA4(fr/m)*	6	0.17b	18.44 ± 1.08a	0.0234 ± 0.0007a
$(NH_4)_2SO_4$*	*CB2(fr)*	6	0.30a	16.70 ± 0.21b	0.0213 ± 0.0014b
$(NH_4)_2SO_4$*	*CB4[fr/(nm)m]*	6	0.19b	18.77 ± 0.67a	0.0240 ± 0.0001a

•*Frankia* and/or mycorrhizal association status after experimentation; ◆months; ▲continuously fed with external ^{14}N from time of transplanting until harvest; ▼deprived of ^{14}N for 4 weeks before harvest; *6 weeks external ^{14}N supply to both N-donor and N-receiver from transplanting for seedling establishment, and then without any N-supply for another 18 weeks until harvest; shoot $\delta^{15}N$ = –6.44 (N-plus experiment) and –4.25 (N-minus experiment) for nodulated casuarinas cultivated in an N-free medium. m: mycorrhizal infected; (nm)m: initially non-mycorrhizal but mycorrhizal colonised through its partner during experiment; fr: *Frankia* nodulated; fr/(nm)m: initially non-mycorrhizal *Frankia* infected but mycorrhizal colonised through its partner during experiment.

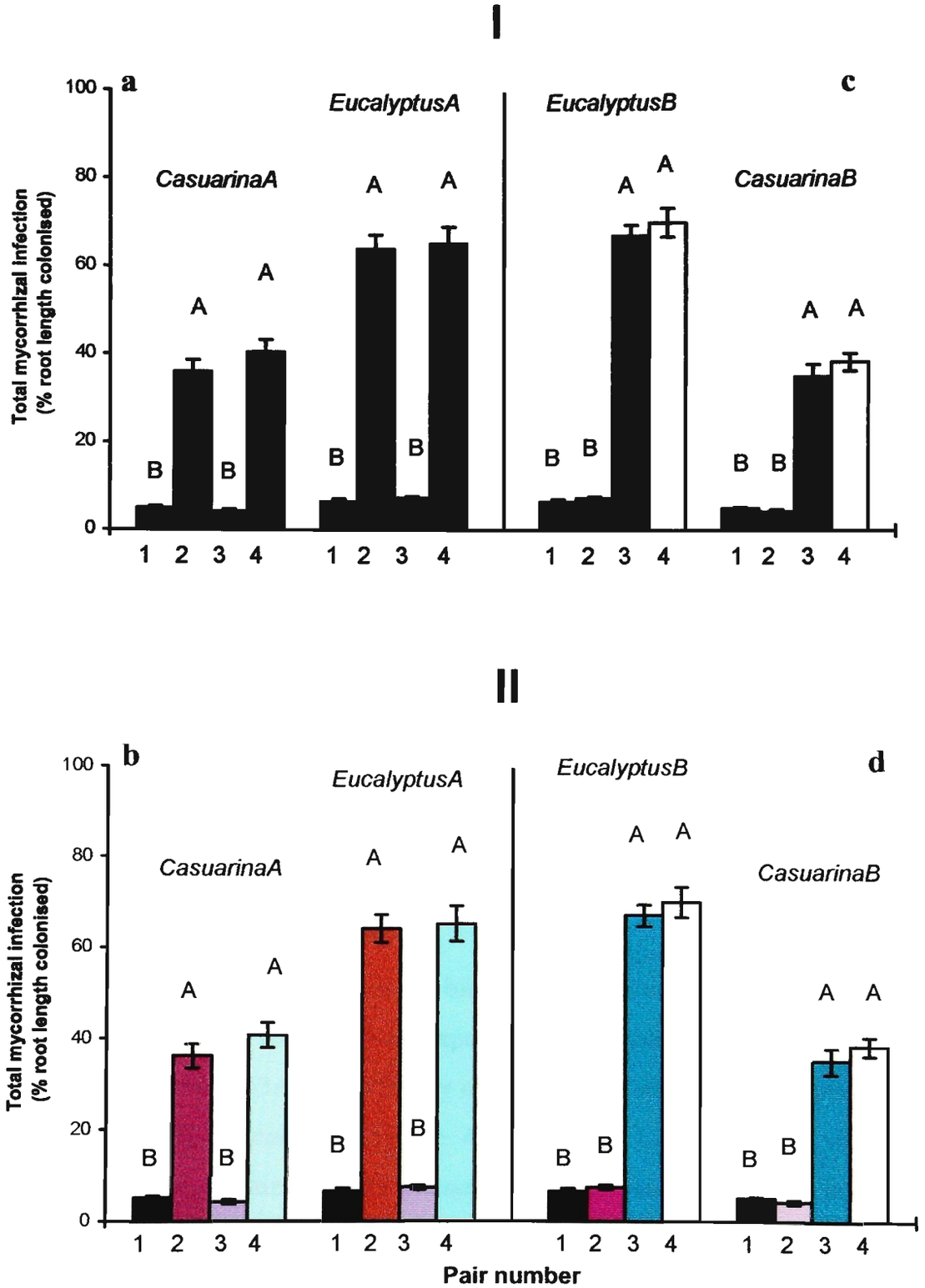

Figure 4.12. Mycorrhizal root colonisations of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a and b), and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (c and d). [Means ± SE, n = 9; different letter (A, B) signifies difference at P = 0.01]. (I and II: with and without external nitrogen).

Shoot $\delta^{15}N$ values were lower but $\%N_{BNF}$ values were higher in the N-plus than in the N-minus plants (Table 4.7). However, the reverse was true for the specific nodule activity, though casuarinas in the N-plus experiment yielded more nodule biomass and N content than those in the N-minus experiment (Inset in Figure 4.15A versus Inset in Figure 4.15B). Shoot $\delta^{15}N$, % N_{BNF} and specific nodule activity differed significantly between non-mycorrhizal and mycorrhizal casuarinas in both the N-plus and the N-minus experiment.

4.3.1.2.3 *Responsiveness of Casuarina and Eucalyptus to mycorrhization*

Casuarina and *Eucalyptus* produced much greater biomass in the N-plus experiment than in the N-minus experiment (see Appendix 2c and 2d). Biomass in casuarinas was greater in the nodulated mycorrhizal > the nodulated non-mycorrhizal > the mycorrhizal > the control plants in the N-plus experiment, whereas no biomass difference existed between the sole mycorrhizal and the control plants in the N-minus experiment. No significant dry matter difference was found between the mycorrhizal *Eucalyptus* in the N-plus experiment (Figure 4.13A), regardless of their partners nodulation status. By contrast, only the mycorrhizal eucalypts, if paired with the nodulated mycorrhizal casuarinas, had a significantly higher biomass than plants grown in the other treatments (Figure 4.13B), indicating a benefit was obtained from the nodulated casuarinas in the N-minus experiment.

No relationship was observed between biomass and mycorrhization in *Casuarina* in both the N-plus and the N-minus experiment, because of the interaction with nodulation (Figures 4.13A and 13B). By contrast, dry matter responded positively to mycorrhization in *Eucalyptus* (Figure 4.13B). Both *Casuarina* and *Eucalyptus* benefited from mycorrhization, but *Casuarina* benefited more because it needs more N, and that is why *Casuarina* responded more obviously in N-plus experiments. Moreover, N_2-fixation had its greatest influence on N accumulation in combination with mycorrhization especially when N was limited.

Nitrogen accumulation in both species was much higher when external N was provided (Figures 4.14A versus 4.14B). Nitrogen content in nodules also increased

(Insets in Figure 4.15). However, for both the N-plus and the N-minus experiment, N content did not show a direct response to mycorrhization in *Casuarina* whereas a positive correlation was found in *Eucalyptus*. This indicated that *Casuarina* satisfied its N requirement through N_2-fixation.

Casuarina and *Eucalyptus* accumulated significantly more N when they were mycorrhizal in the N-plus experiment (Figure 4.14A). Nitrogen content in the nodulated mycorrhizal *Casuarina* plants was greater than that in the nodulated non-mycorrhizal *Casuarina*; the latter had a higher N content than the sole mycorrhizal *Casuarina* due to N_2-fixation. This suggested that the dual *Frankia*/mycorrhizal colonisation was the greatest determinant of N accumulation in casuarinas. The mycorrhizal eucalypts showed no N content difference, regardless of pairing with nodulated or non-nodulated casuarinas.

In the N-minus experiment, the single mycorrhizal *Casuarina* and *Eucalyptus* exhibited a similar amount of N accumulation to that of the non-mycorrhizal controls (Figure 4.14B). In contrast, N content of the nodulated mycorrhizal *Casuarina* plants was greater than that of the non-nodulated mycorrhizal casuarinas. *Eucalyptus* displayed its highest N content when partnered with the dual *Frankia*/mycorrhizal colonised casuarinas. The results suggested that not only could the dual *Frankia*/mycorrhizal inoculation enhance N accumulation in casuarinas, but also that N had been translocated to their pairing partners when adequate N was available.

Dry matter production correlated with N accumulation and increased with increasing N content in both *Casuarina* and *Eucalyptus* in both the N-plus and the N-minus experiments (Figures 4.15A-B). The response was highest in the nodulated mycorrhizal pairings. In addition, nodule biomass also linearly correlated with N content (Insets in Figures 4.15A-B). Although the nodulated mycorrhizal casuarinas had similar nodule N concentrations (%) (data not shown), the N content was significantly different from the nodulated non-mycorrhizal plants, because of higher nodule dry matter yields.

4.3.1.2.4 *Relationship between N accumulation and $\delta^{15}N$ value*

$\delta^{15}N$ values decreased with increasing N concentration (Figures 4.16A-B) or N content in the plants (Figures 4.17A-B). In general, $\delta^{15}N$ values were more closely correlated with N accumulation in *Casuarina* than in *Eucalyptus*. *Casuarina* had generally lower $\delta^{15}N$ values than *Eucalyptus* (Figures 4.16-4.17). $\delta^{15}N$ values varied with the external N input in both species. *Casuarina* had lower shoot $\delta^{15}N$ values and higher root $\delta^{15}N$ values in the N-plus than in the N-minus experiment (Figure 4.16 versus Figure 4.17). This was probably due to the nodules which had a higher average $\delta^{15}N$ value of 2.13 in the N-plus experiment and an average $\delta^{15}N$ value of 1.52 in the N-minus experiment. On the other hand, *Eucalyptus* had higher $\delta^{15}N$ values in both shoot and root in the N-plus experiment (Figure 4.16 versus Figure 4.17).

4.3.1.2.5 *Relationship between N accumulation and leaf chlorophyll content*

Leaf chlorophyll content positively depended on N content and was highest in the dual nodulated mycorrhizal *Casuarina/Eucalyptus* pairs (Figure 4.18). The nodulated non-mycorrhizal casuarinas had a higher chlorophyll content than the non-mycorrhizal casuarinas, but not between the sole mycorrhizal and the non-mycorrhizal casuarinas, nor between the sole mycorrhizal and the non-mycorrhizal eucalypts.

4.3.1.2.6 *Amino acid composition in xylem sap of Casuarina and Eucalyptus*

In general, it was difficult to find a consistent shoot or root amino acid pattern between treatments in both species (Figure 4.19). Total amino acids were highest in the casuarinas when nodulated and mycorrhizal, less so when non-nodulated but mycorrhizal and lowest when non-mycorrhizal. The mycorrhizal eucalypts growing with the nodulated mycorrhizal casuarinas, had significantly higher total amino acid contents than the other *Eucalyptus* plants. No asparagine, aspartate, proline or citrulline was detected in *Eucalyptus* plants. In general, it was interesting that nodulated casuarinas had much higher proline and citrulline contents than the sole mycorrhizal ones, which in turn had higher contents than the non-mycorrhizal control plants.

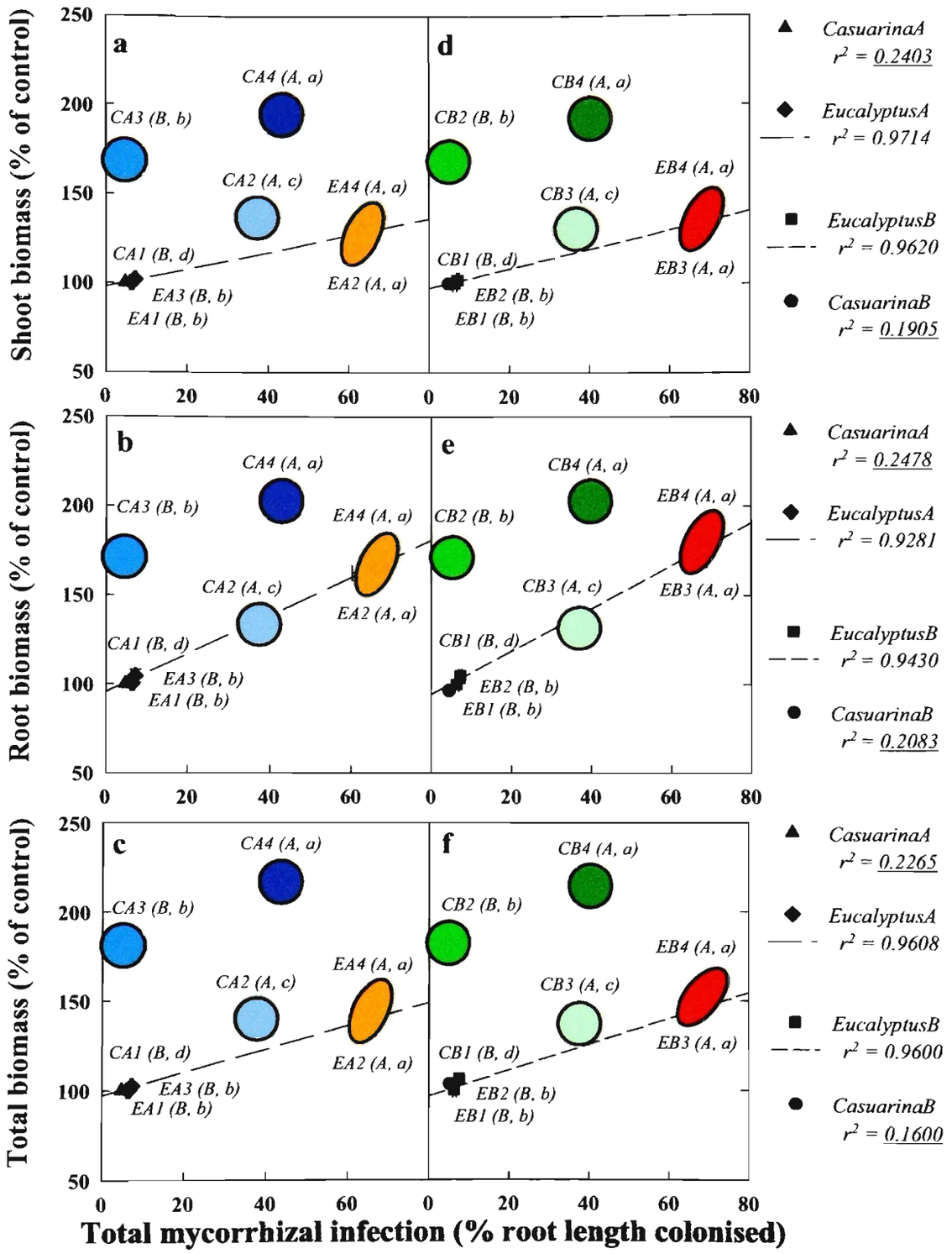

Figure 4.13-A. Relationships between root mycorrhizal colonisation and tissue biomass production of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a-c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d-f). N-plus experiment. [Means ± SE, n = 9; letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

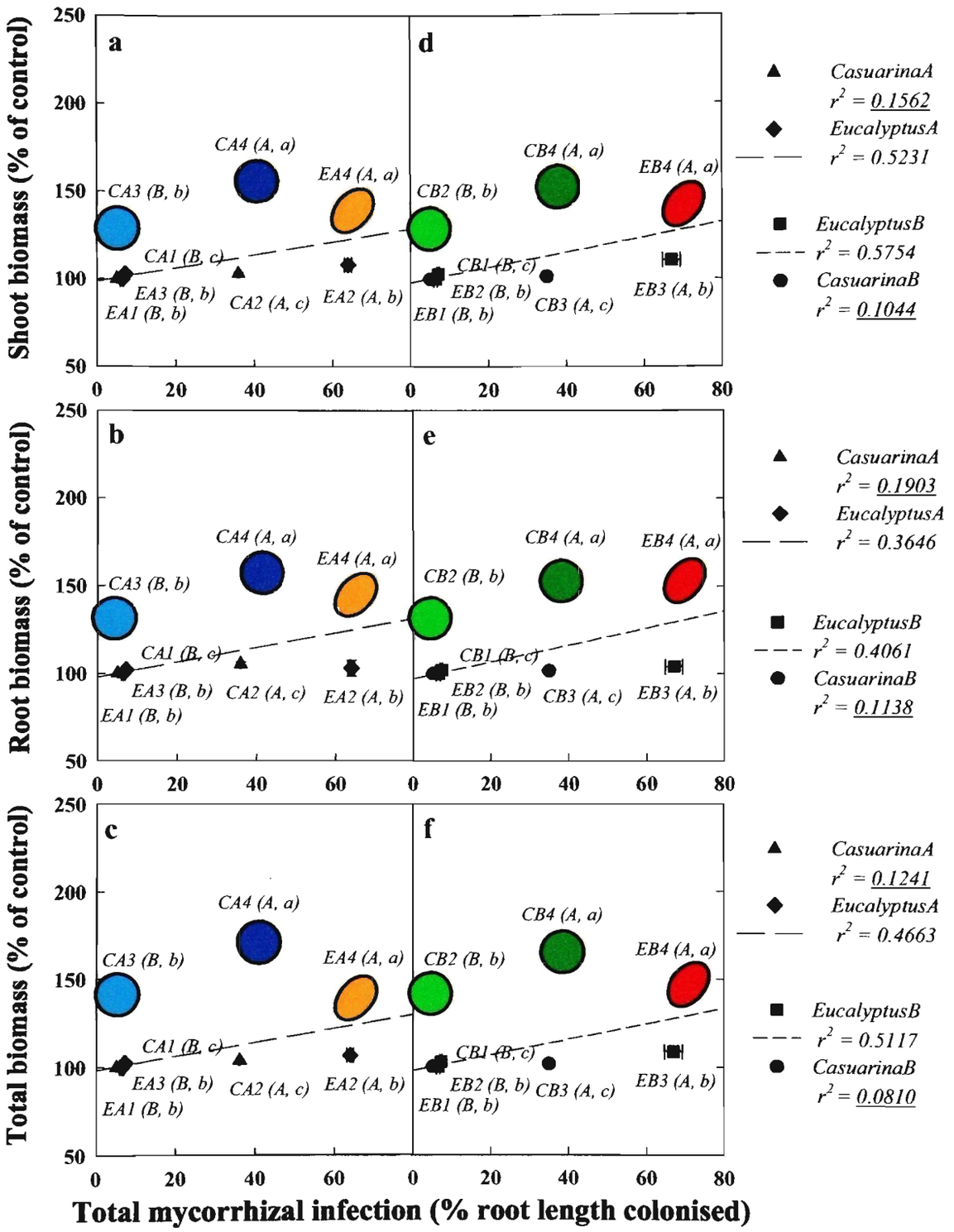

Figure 4.13-B. Relationships between root mycorrhizal colonisation and tissue biomass production of *CasuarinaA*(N-donor) and *EucalyptusA* (N-receiver) (a-c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d-f). N-minus experiment. [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

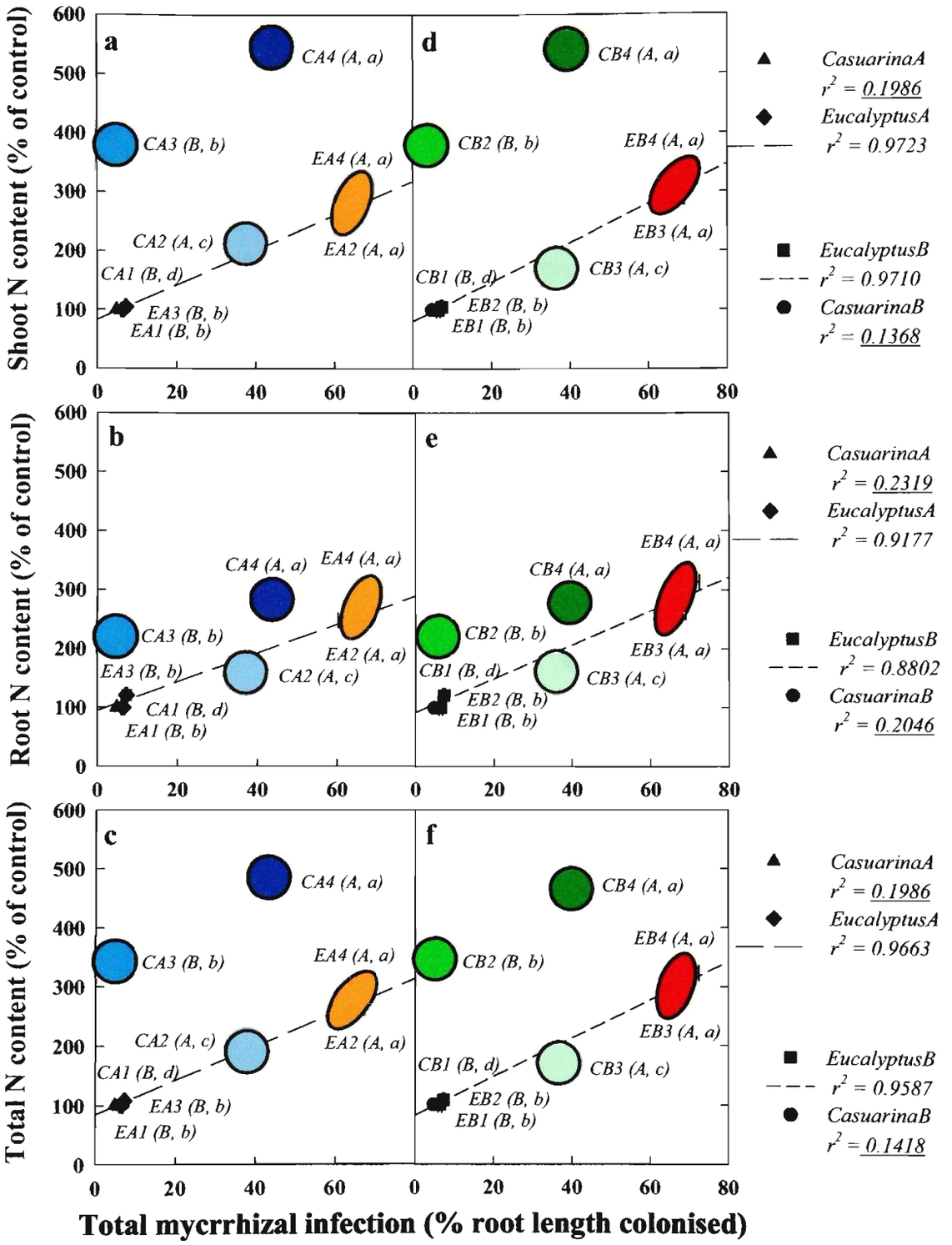

Figure 4.14-A. Relationships between root mycorrhizal colonisation and tissue N content of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a-c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d-f). N-plus experiment. [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

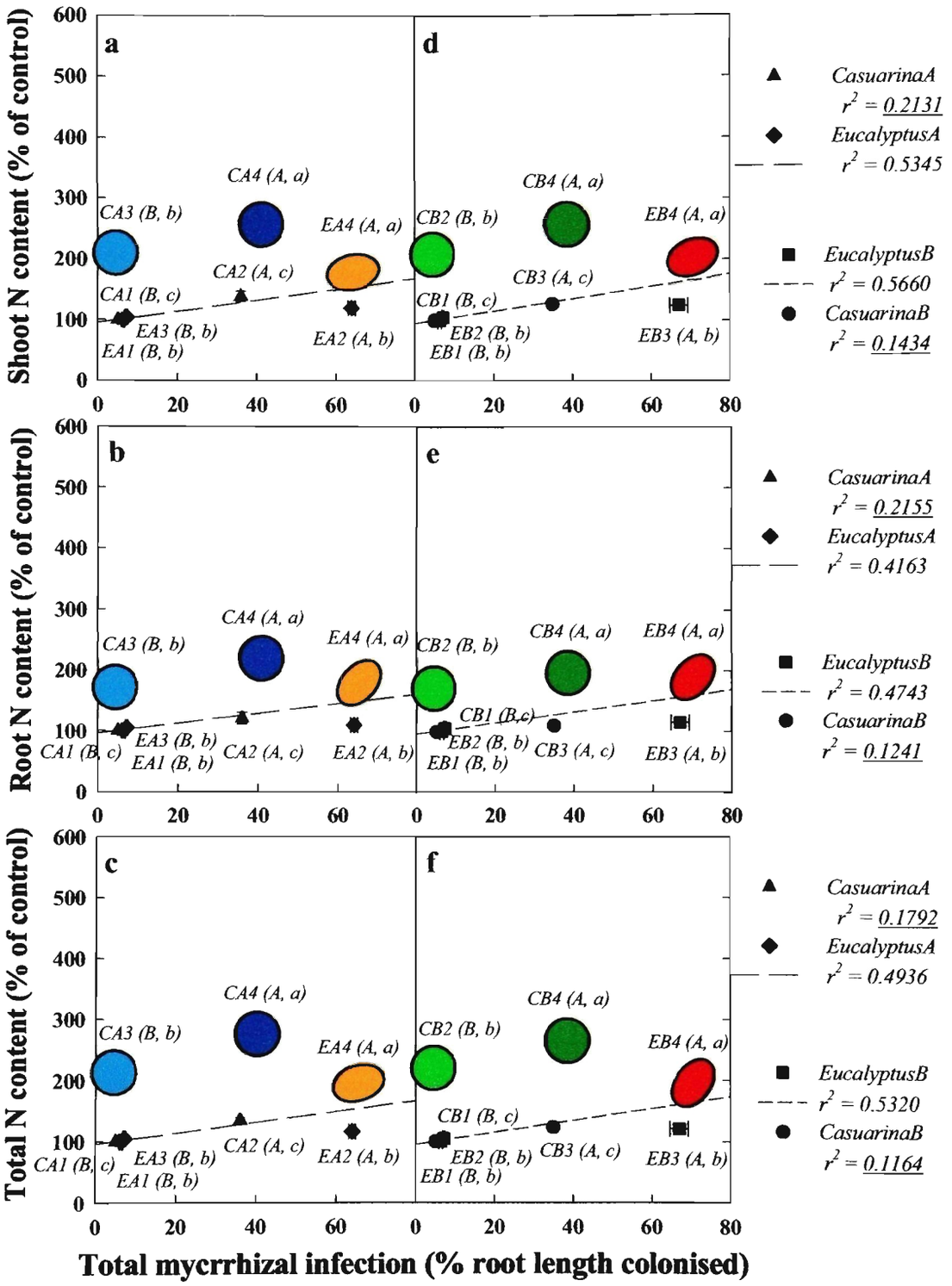

Figure 4.14-B. Relationships between root mycorrhizal colonisation and tissue N content of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a-c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d-f). N-minus experiment. [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x*, *y* parameters, respectively].

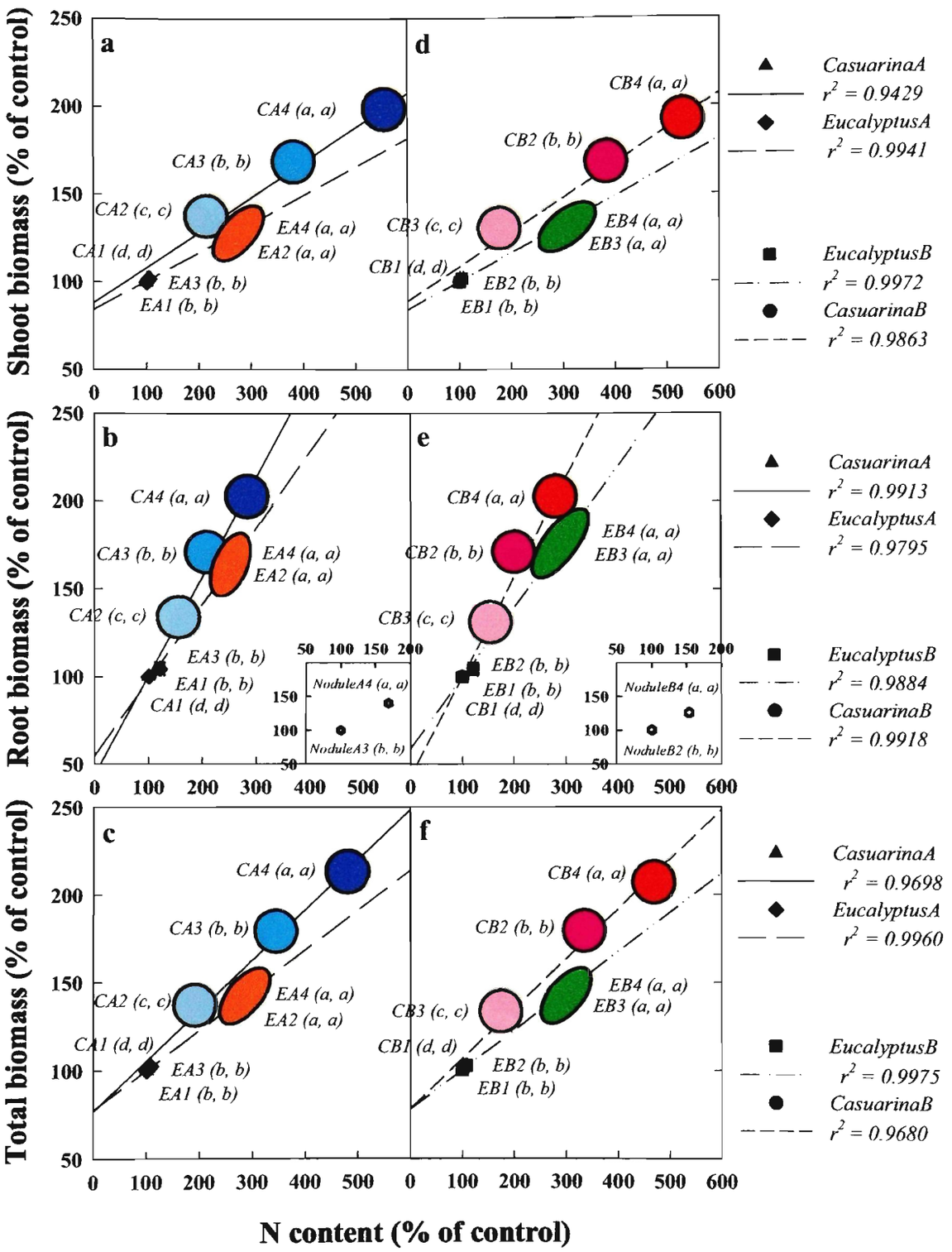

Figure 4.15-A. Relationships between tissue N content and biomass production of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a-c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d-f). N-plus experiment. [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

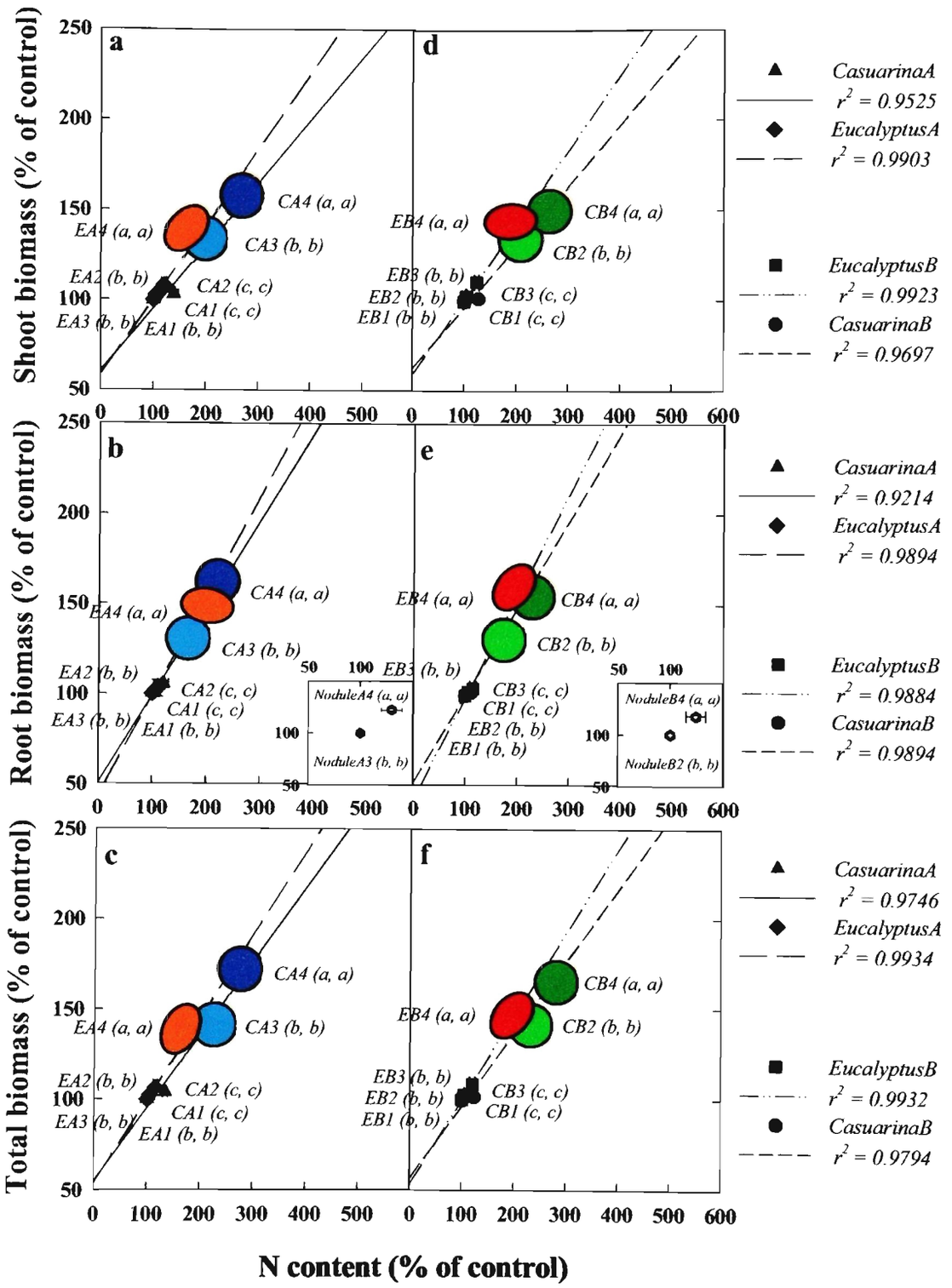

Figure 4.15-B. Relationships between tissue N content and biomass production of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a-c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d-f). N-minus experiment. [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

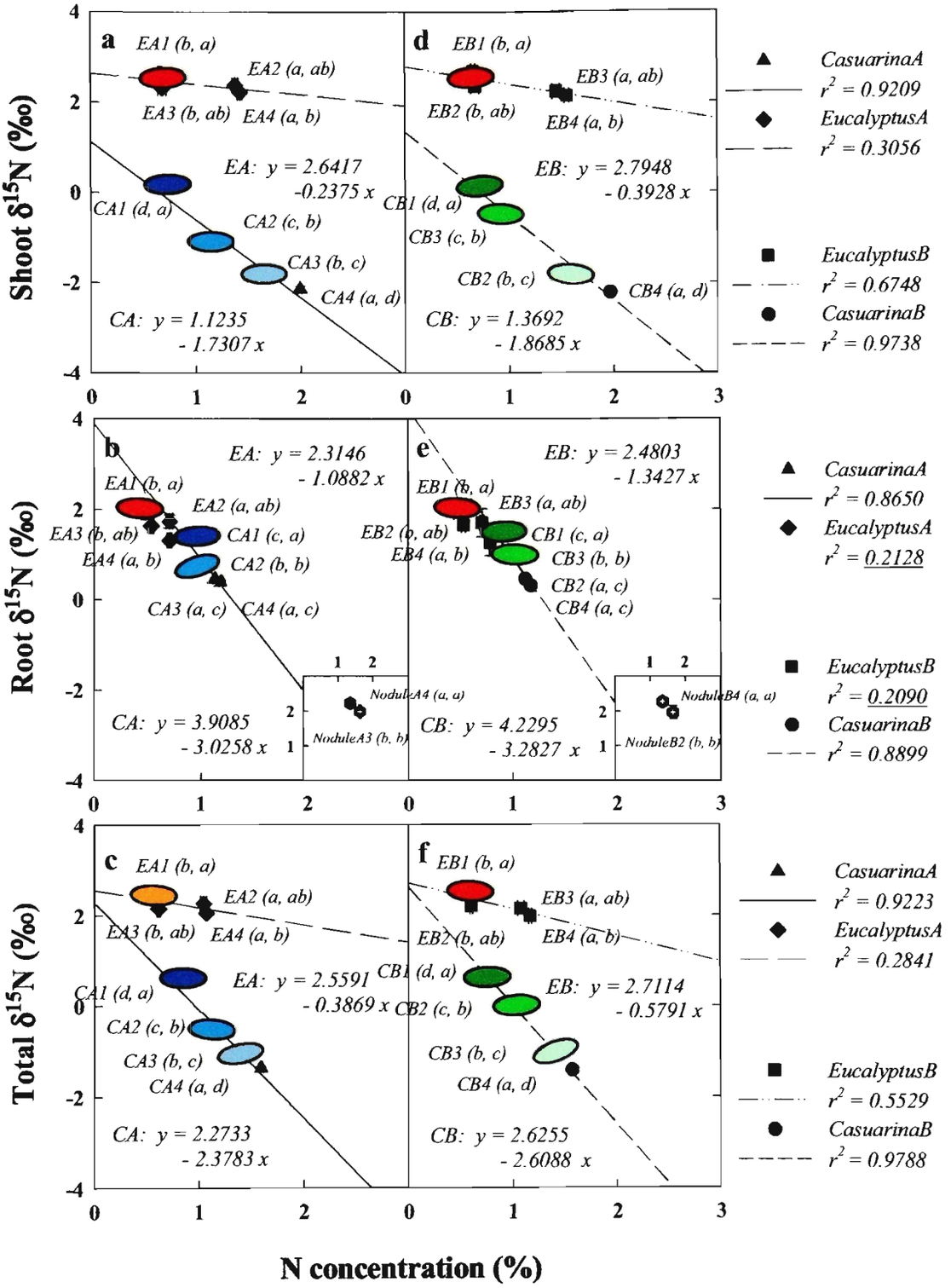

Figure 4.16-A. Relationships between N concentration and $\delta^{15}N$ value of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a-c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d-f). N-plus experiment. [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

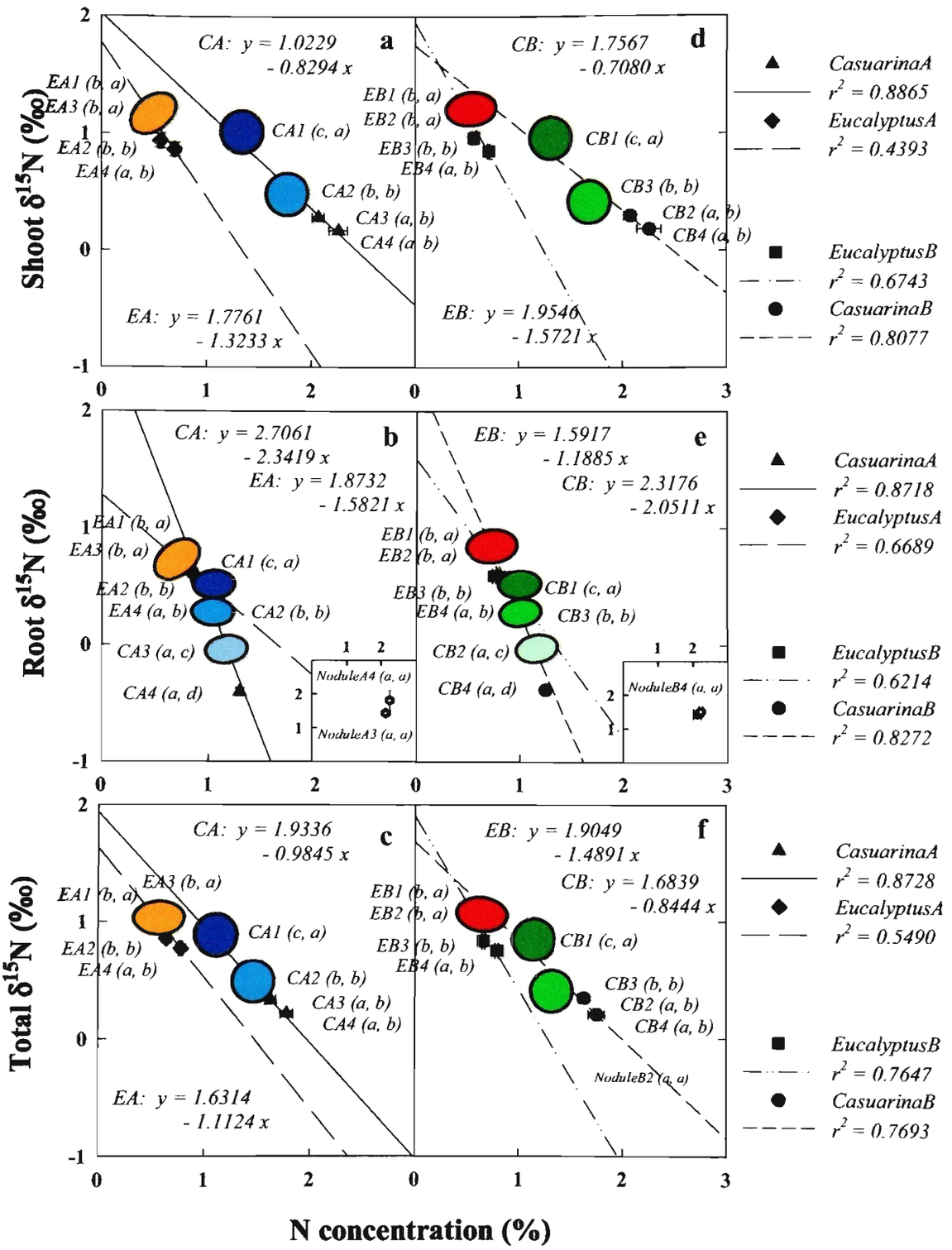

Figure 4.16-B. Relationships between N concentration and $\delta^{15}N$ value of ***CasuarinaA*** (N-donor) and ***EucalyptusA*** (N-receiver) (a-c); and ***EucalyptusB*** (N-donor) and ***CasuarinaB*** (N-receiver) (d-f). N-minus experiment. [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

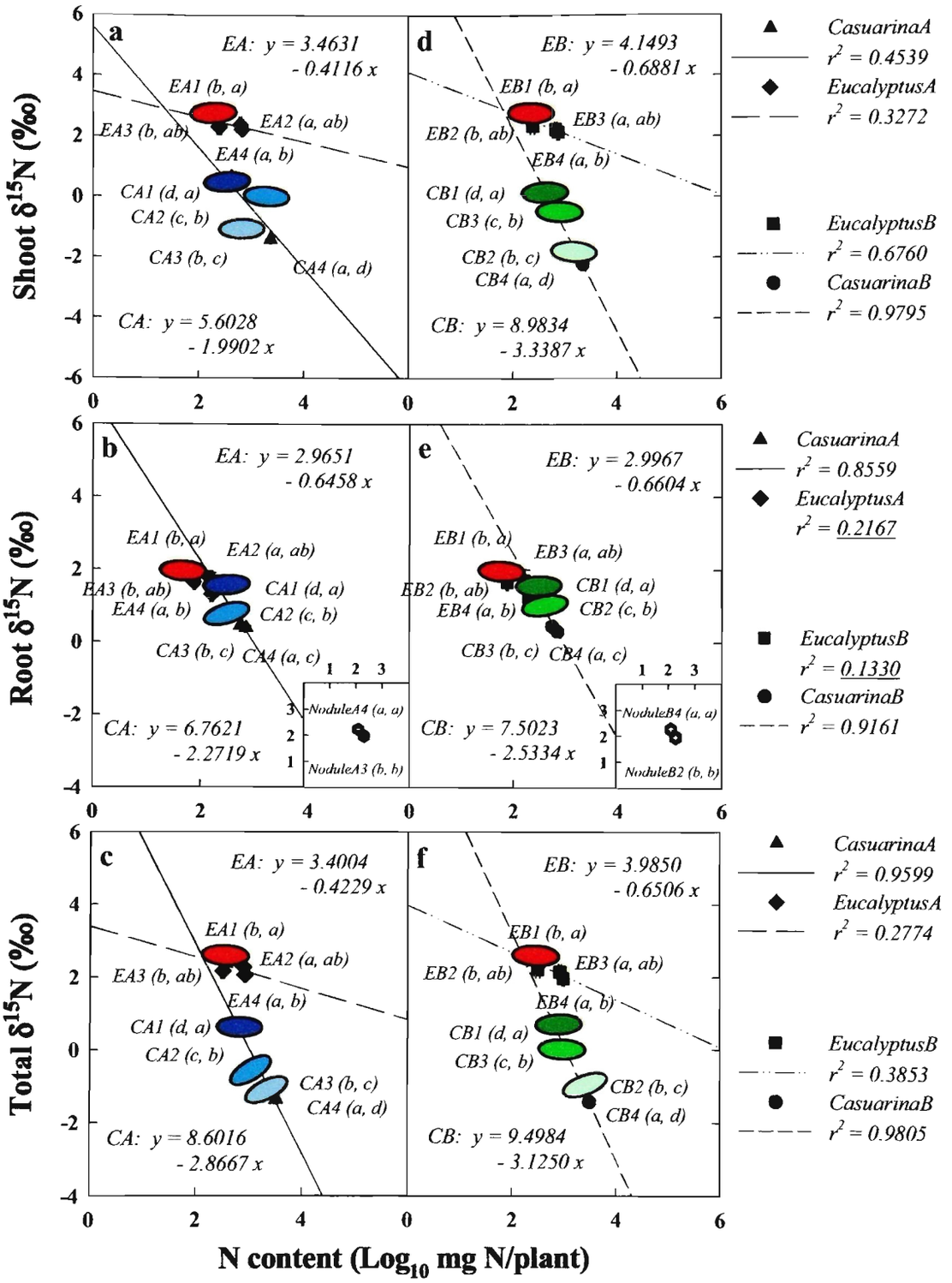

Figure 4.17-A. Relationships between N content and δ^{15}N value of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a - c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d - f). N-plus experiment. [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

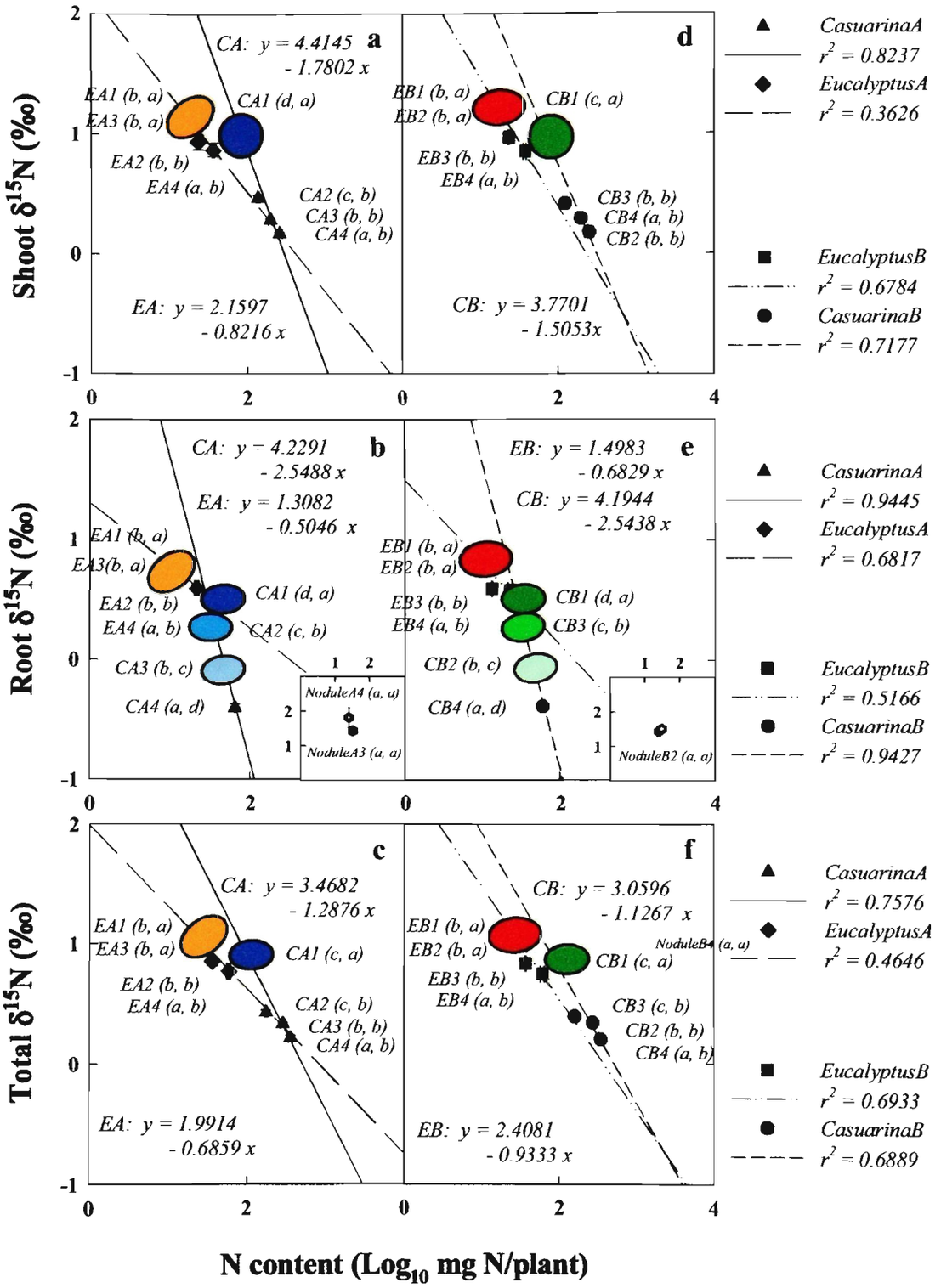

Figure 4.17-B. Relationships between N content and $\delta^{15}N$ value of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a - c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d - f). N-minus experiment. [Means±SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

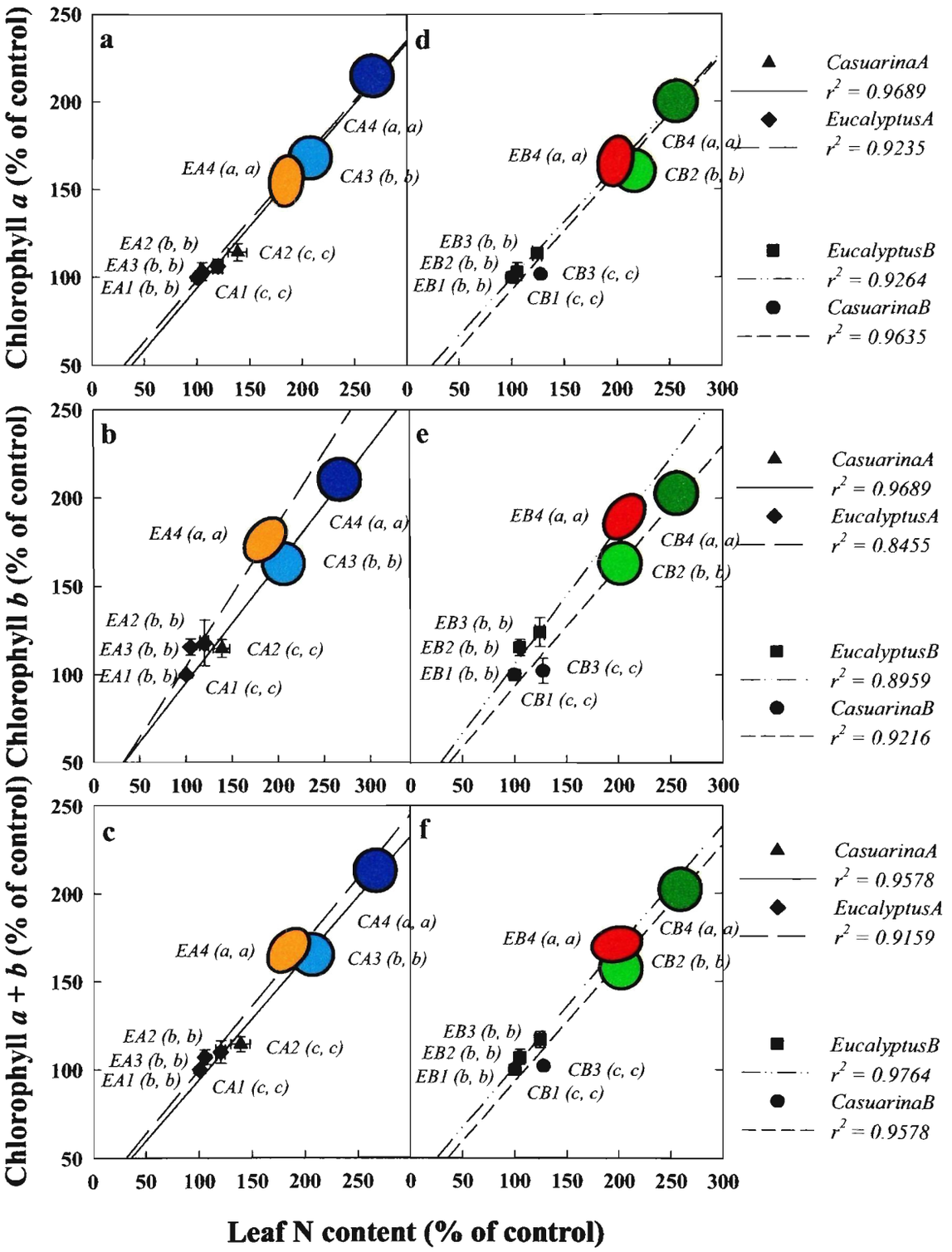

Figure 4.18. Relationships between leaf N content and chlorophyll *a*, *b* and *a* + *b* of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a-c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d - f). N-minus experiment. [Means ± SE, n = 9; different letter (*a*, *a*) signifies difference at P = 0.05 for *x*, *y* parameters, respectively].

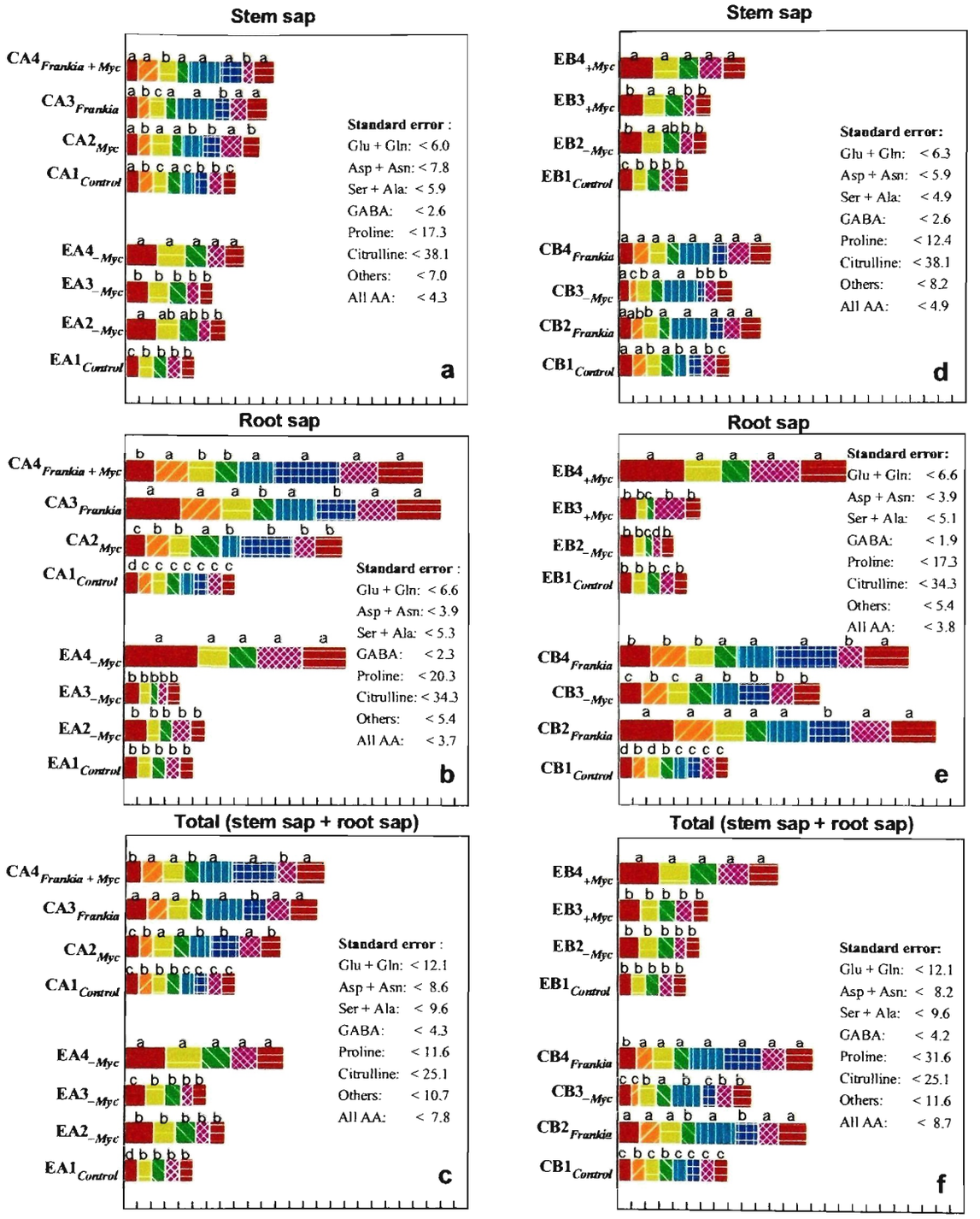

Figure 4.19. Amino acid composition of *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (a-c); and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (d-f). N-minus experiment. [Means ± SE, n = 9; different letter (*a*, *a*) signifies difference at P = 0.05 for the same amino acid, respectively].

4.3.1.2.7 *Nitrogen transfer between Casuarina and Eucalyptus*

Table 4.8. N-transfer between *CasuarinaA* (N-donor) and *EucalyptusA* (N-receiver) (A), and *EucalyptusB* (N-donor) and *CasuarinaB* (N-receiver) (B). ^{14}N was continuously fed to both N-donor and N-receiver from time of transplanting until harvest, except 4 weeks N deprivation to the N-receiver before harvest in the N-plus experiment (12-months-old seedlings). ^{14}N was fed to both N-donor and N-receiver for 6 weeks from transplanting for seedling establishment, then withheld for another 18 weeks till harvest in the N-minus experiment (6-months-old seedlings) [Values are Means, n = 9, different letter (a, b) signifies difference at P = 0.05].

Treatments	N-transfer											
	$\delta^{15}N$ (‰)*			% $N_{transfer}$			$N_{transfer}$ (mg/plant)			% NDFT		
	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total
I: N-transfer from *CasuarinaA* (N-donor) to *EucalyptusA* (N-receiver)												
A. N-plus experiment												
CA4→EA4	2.23b	1.31b	2.05b	15.22a	32.78a	24.00a	120.54a	80.10a	200.64a	18.26a	48.96a	33.61a
CA2→EA2	2.39ab	1.74ab	2.26ab	9.35b	11.39b	10.37b	64.47b	19.42b	83.89b	10.32b	13.27b	11.80b
CA1→EA1	2.64a	1.95a	2.49a	—	—	—	—	—	—	—	—	—
B. N-minus experiment												
CA4→EA4	0.88b	0.60b	0.78b	22.91a	21.38a	22.08a	10.17a	5.98a	16.15a	29.76a	27.20a	28.48a
CA2→EA2	0.95b	0.69ab	0.86ab	16.47b	9.43b	12.95b	4.52b	1.33b	5.85b	19.98b	10.43b	15.21b
CA1→EA1	1.13a	0.76a	0.99a	—	—	—	—	—	—	—	—	—
II: N-transfer from *EucalyptusB* (N-donor) to *CasuarinaB* (N-receiver)												
A. N-plus experiment												
EB4→CB4	-2.19c	0.33c	-1.39c	27.26a	26.20a	26.74a	283.64a	70.06a	353.70a	12.57a	10.16a	11.37a
EB3→CB3	-0.45b	1.01b	0.06b	8.93b	12.05b	10.99b	66.28b	22.48b	88.76b	9.10b	5.73b	7.42b
EB1→CB1	0.16a	1.44a	0.64a	—	—	—	—	—	—	—	—	—
B. N-minus experiment												
EB4→CB4	0.19c	-0.38c	0.21c	18.69a	31.43b	25.06a	9.10a	10.81a	19.91a	3.54a	17.87a	10.71a
EB3→CB3	0.42b	0.28b	0.39b	13.65b	10.14a	11.90b	3.84b	1.53b	5.37b	3.02a	4.63b	3.83b
EB1→CB1	0.95a	0.50a	0.85a	—	—	—	—	—	—	—	—	—

*$\delta^{15}N$ values of N-receiver; shoot and root $\delta^{15}N$ values = –6.44 and –4.56 (N-plus experiment), and –4.25 and –2.42 (N-minus experiment) for nodulated *Casuarina* and *Eucalyptus* cultivated in an N-free medium.

Table 4.8 shows that N was translocated from the mycorrhizal *Casuarina* to the mycorrhizal *Eucalyptus* and vice versa in both the N-plus and the N-minus experiments. The total amount of N-transferred in the N-plus experiment was higher than that in the N-minus experiment. Mycorrhizal *Eucalyptus* delivered much more N to the nodulated mycorrhizal than to the non-nodulated mycorrhizal *Casuarina,* especially when adequate external N was accessible.

4.3.2 N-transfer between pairs of soybean and *Sorghum* plants (N-minus experiment)

4.3.2.1 *Experimental Design*

Table 4.9 shows the experimental design for N-transfer between soybean and *Sorghum*.

Table 4.9. Pairings of soybean (cv. Manark) and *Sorghum* (cv. New Nudgee) to identify N-transfer between species. Both N-donor and N-receiver were continuously fed with ^{14}N nutrition for 3 weeks for plant establishment from sowing, and then left without N-supply for another 9 weeks until harvest.

N-donor		N-receiver	Code
A. N-transfer from soybean to *Sorghum*			
Pair 1: soybean$A_{control}$	+	*Sorghum*$A_{control}$	soyA1→SorA1
Pair 2: soybeanA_{AM}	+	*Sorghum*A_{-AM}	soyA2→SorA2
Pair 3: soybean$A_{rhizobia}$	+	*Sorghum*A_{-AM}	soyA3→SorA3
Pair 4: soybean$A_{rhizobia + AM}$	+	*Sorghum*A_{-AM}	soyA4→SorA4
B. N-transfer from *Sorghum* to soybean			
Pair 1: *Sorghum*$B_{control}$	+	soybean$B_{control}$	SorB1→soyB1
Pair 2: *Sorghum*B_{-AM}	+	soybean$B_{rhizobia}$	SorB2→soyB2
Pair 3: *Sorghum*B_{AM}	+	soybeanB_{-AM}	SorB3→soyB3
Pair 4: *Sorghum*B_{AM}	+	soybean$B_{rhizobia}$	SorB4→soyB4

Table 4.10. $\delta^{15}N$ values, percentage of biological nitrogen fixation ($\%N_{BNF}$) and specific nodule activity (mg N fixed/mg nodule dry weight) in 3-months-old soybeanA and soybeanB [Means ± SE, n = 9; different letter (a, b) signifies difference at P = 0.05].

N-source▲	Treatment●	Age	$\delta^{15}N$ (‰)	% N_{BNF}	Specific nodule activity
$(NH_4)_2SO_4$	soyA3*(rhi)*	12wks	6.01a	31.69±1.10b	0.0514 ± 0.0007b
$(NH_4)_2SO_4$	soyA4*(rhi/m)*	12wks	5.35b	36.69±3.59a	0.0537 ± 0.0021a
$(NH_4)_2SO_4$	soyB2*(rhi)*	12wks	5.98a	32.11±1.32b	0.0514 ± 0.0011b
$(NH_4)_2SO_4$	soyB4[*rhi/(nm)m*]	12wks	5.26b	39.33±2.70a	0.0535 ± 0.0013a

▲external ^{14}N was continuously fed to both N-donor and N-receiver for 3 weeks for plant establishment from sowing; they were then left without N-supply for another 9 weeks until harvest; ●*Rhizobium* and/or mycorrhizal association status after experiment. Shoot $\delta^{15}N$ = 2.35‰ for nodulated soybean cultivated in an N-free medium. m: mycorrhizal infected; (nm)m: initially non-mycorrhizal but mycorrhizal colonised through its partner during experiment; rhi: *Rhizobium* nodulated; rhi/(nm)m: initially non-mycorrhizal *Rhizobium* infected but mycorrhizal colonised through its partner during experiment.

4.3.2.2 *Results*

4.3.2.2.1 *Formation of common arbuscular mycorrhizal networks*

The non-mycorrhizal pairings in both species were mainly uncolonised. In contrast, all the plants inoculated with the AM fungus *Glomus mosseae* developed mycorrhization during the glasshouse cultivation. The originally non-inoculated plants became infected through their mycorrhizal partners at the end of the experiment (Figure 4.20). Mycorrhizal infection differed significantly between the mycorrhizal and the non-mycorrhizal plants in both species, but there also was a significant difference between soybean and *Sorghum*. Together with the observations by the light and the Environmental Scanning Electron Microscopes, the results indicated both species were successfully colonised by *G. mosseae* and a common underground AM link had been established between the soybean and *Sorghum* roots.

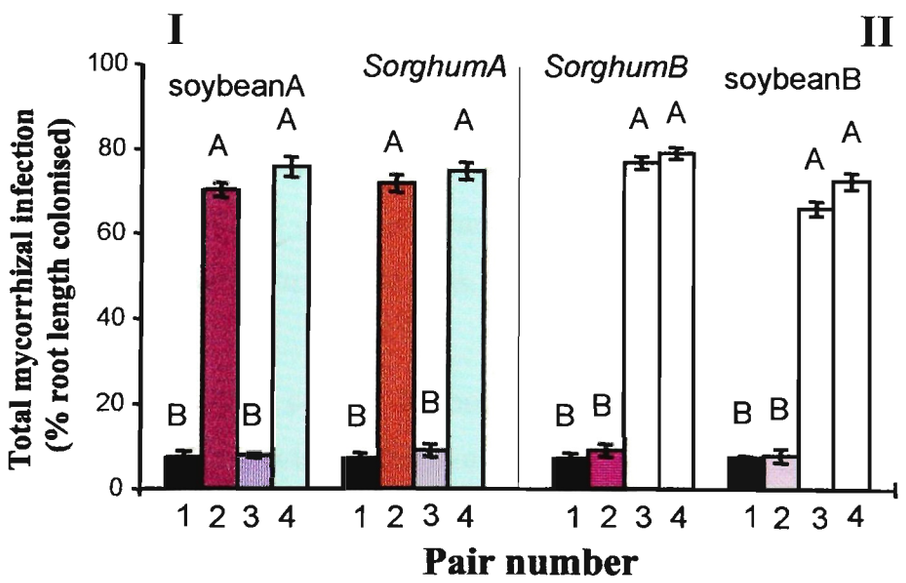

Figure 4.20. Mycorrhizal root colonisations of soybeanA (N-donor) and *SorghumA* (N-receiver) (I), and *SorghumB* (N-donor) and soybeanB (N-receiver) (II). [Means ± SE, n = 9, different letter (A, B) signifies difference at P = 0.01].

4.3.2.2.2 *Biological N_2-fixation in soybeans*

Table 4.10 shows that shoot $\delta^{15}N$ values, $\%N_{BNF}$ and specific nodule activity in the nodulated mycorrhizal soybeans differed significantly from those in the nodulated, non-mycorrhizal soybeans when no suitable external N source was available, and that they were independent of the source of mycorrhization.

4.3.2.2.3 *Responsiveness of soybean and Sorghum to mycorrhization*

Due to N_2-fixation with *Rhizobium*, biomass did not respond to AM mycorrhization in soybean. In contrast, shoot, root and total dry matter yield correlated positively with root colonisation in *Sorghum* (Figure 4.21). The nodulated soybeans, regardless of their mycorrhizal status, had significantly more biomass than the sole mycorrhizal ones, which in turn had more biomass than the control soybeans. On the other hand, mycorrhization increased dry weight in *Sorghum*, being highest in the mycorrhizal *Sorghum* that grew with the nodulated mycorrhizal soybeans.

No correlation between N content and mycorrhization was found in soybean, but a positive relationship existed in *Sorghum* (Figure 4.22). Nitrogen accumulation showed the following pattern: the nodulated (either mycorrhizal or non-mycorrhizal) > the non-nodulated mycorrhizal > the control soybeans. On the other hand, the mycorrhizal *Sorghum* had significantly higher N content than the non-mycorrhizal ones, and the mycorrhizal *Sorghum* reached its highest N accumulation, if grown with the nodulated mycorrhizal soybeans.

Biomass increased with N content for both soybean and *Sorghum* (Figure 4.23) and the response in biomass with N content was as follows: the nodulated > the sole mycorrhizal > the control soybeans when they served as the N-donors; whereas the sole mycorrhizal soybeans had a similar response as the controls in the N-receivers. The mycorrhizal *Sorghum* had a higher response than the non-mycorrhizal ones, and the highest response was in those grown with the nodulated mycorrhizal soybeans. The result suggested that it was *Rhizobium* nodulation, not the AM colonisation, that determined biomass and N accumulation in soybean. The nodulated mycorrhizal soybean had a significant effect on growth performance of its adjacent *Sorghum*.

4.3.2.2.4 *Relationship between N accumulation and $\delta^{15}N$ value*

$\delta^{15}N$ values in shoot, root and total were negatively related to N concentration (Figure 4.24) and N content (Figure 4.25), and the correlation between $\delta^{15}N$ values and N concentration or N content was closer in soybean than in *Sorghum*.

$\delta^{15}N$ values in shoot, root and total showed a consistent variance for both soybean and *Sorghum* (Figures 4.24 and 4.25). Significant $\delta^{15}N$ values differences ranked as follows: the non-nodulated non-mycorrhizal control > the sole mycorrhizal > the nodulated non-mycorrhizal > the nodulated mycorrhizal for soybeans; whereas for *Sorghum* the non-mycorrhizal controls > these grown with the sole *Rhizobium* or mycorrhizal soybean > these paired with the nodulated mycorrhizal soybeans. Along with its partner *Sorghum*, $\delta^{15}N$ values in the nodulated mycorrhizal soybeans showed a more profound response with the dual *Rhizobium*/mycorrhizal symbioses.

4.3.2.2.5 *Relationship between leaf N accumulation and chlorophyll content*

Leaf chlorophyll was highly correlated linearly with leaf N accumulation in both soybean and *Sorghum* (Figure 4.26). The nodulated mycorrhizal soybean/*Sorghum* pairs reached the highest chlorophyll contents, while the sole mycorrhizal soybean/*Sorghum* pairs had significantly higher leaf chlorophyll than the non-mycorrhizal soybean/*Sorghum* control pairs.

4.3.2.2.6 *Amino acid composition in the xylem sap of soybean and Sorghum*

The total amino acids (ureides were not analysed) differed significantly between the sole mycorrhizal and the control soybeans, when they served as the N-donors, but were similar when they served as the N-receivers (Figure 4.27). Both the total amino acids and the asparagine+aspartate content reached their highest value in the nodulated mycorrhizal soybeans. This result suggested that the N fixed by soybean had been integrated into different N-containing organic compounds. On the other hand, mycorrhizal *Sorghum* had a significantly higher total amino acid content (Figure 4.27). The highest amino acid contents were seen in those plants grown with the nodulated mycorrhizal soybeans, indicating that the nodulated AM soybeans could have a significant effect on the sorghum's amino acid composition.

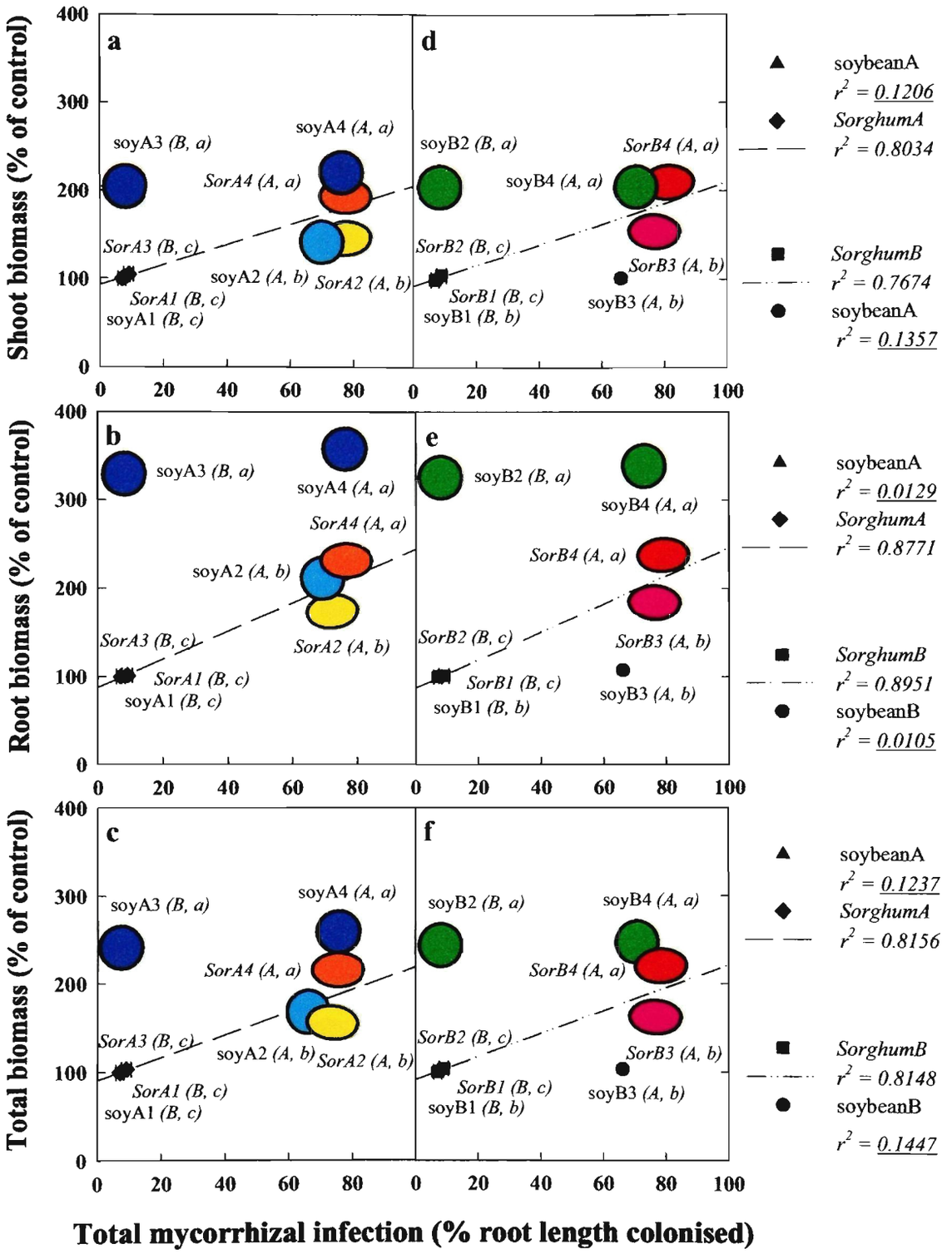

Figure 4.21. Relationships between root mycorrhizal colonisation and tissue biomass production of soybeanA (N-donor) and *SorghumA* (N-receiver) (a-c); and *SorghumB* (N-donor) and soybeanB (N-receiver) (d-f). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

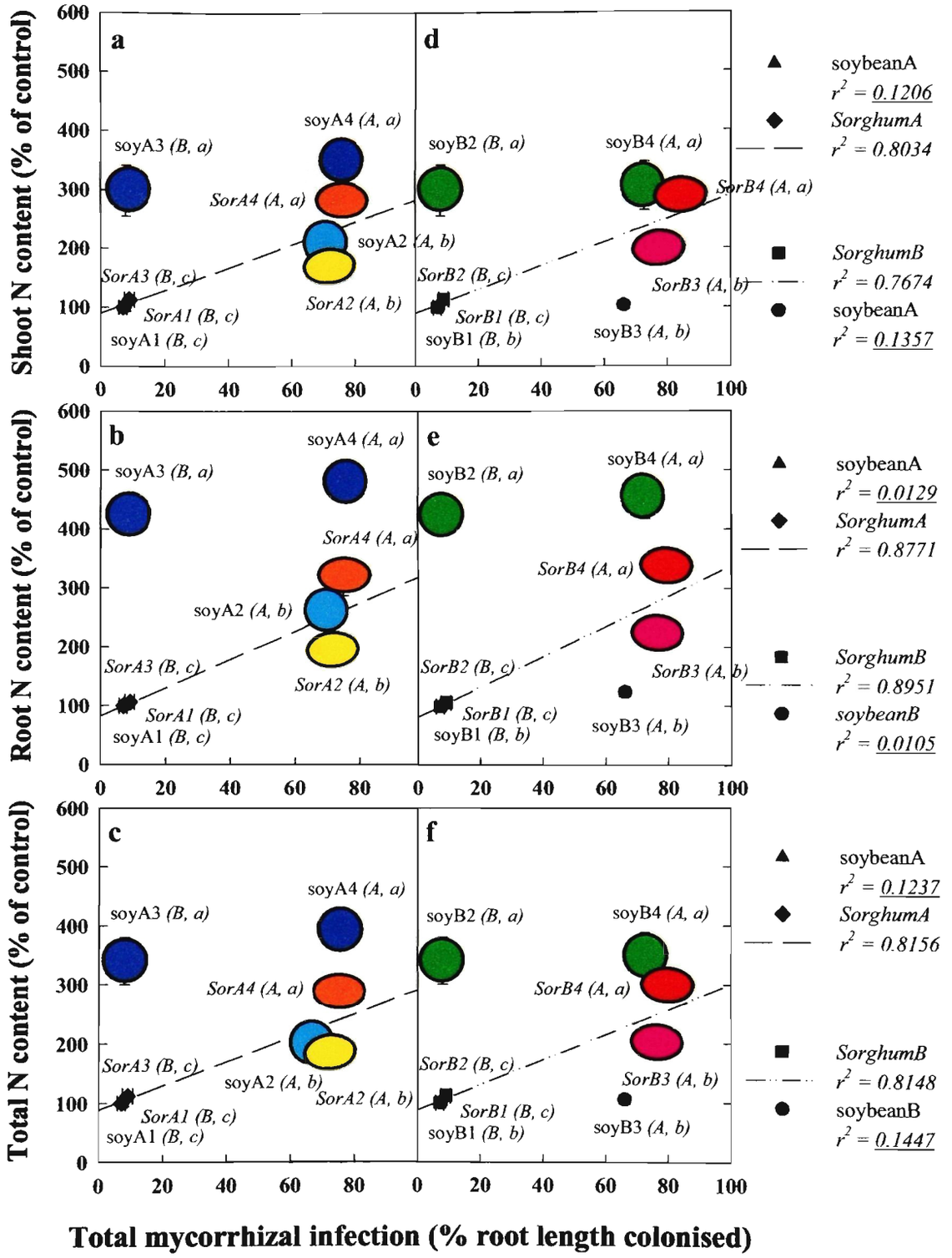

Figure 4.22. Relationships between root mycorrhizal colonisation and tissue N content of soybeanA (N-donor) and *SorghumA* (N-receiver) (a-c); and *SorghumB* (N-donor) and soybeanB (N-receiver) (d-f). [Means ± SE, n = 9; different letter (*A, a*) signifies difference at P = 0.01 or 0.05 for *x, y* parameters, respectively].

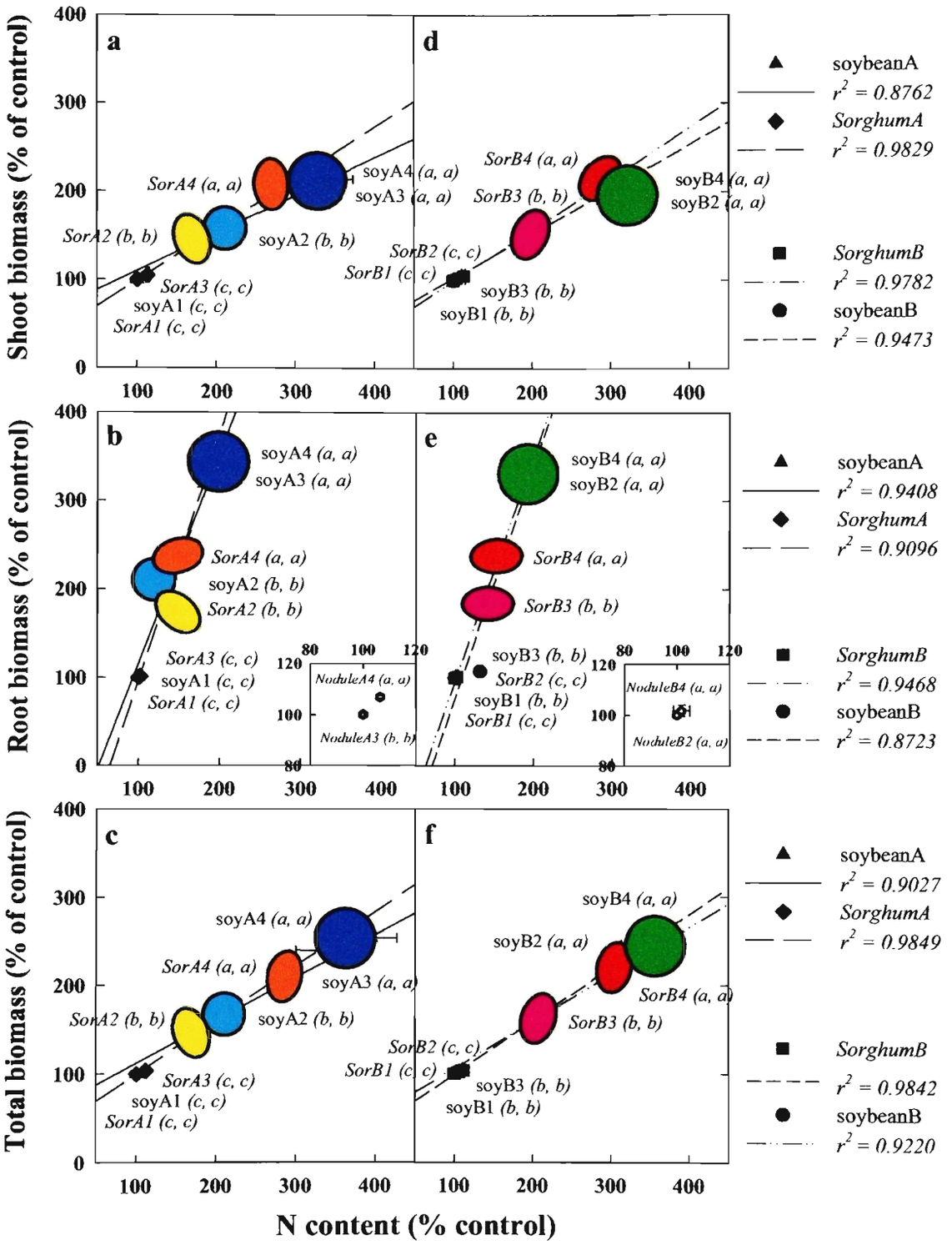

Figure 4.23. Relationships between tissue N content and biomass production of soybeanA (N-donor) and *SorghumA* (N-receiver) (a-c); and *SorghumB* (N-donor) and soybeanB (N-receiver) (d-f). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

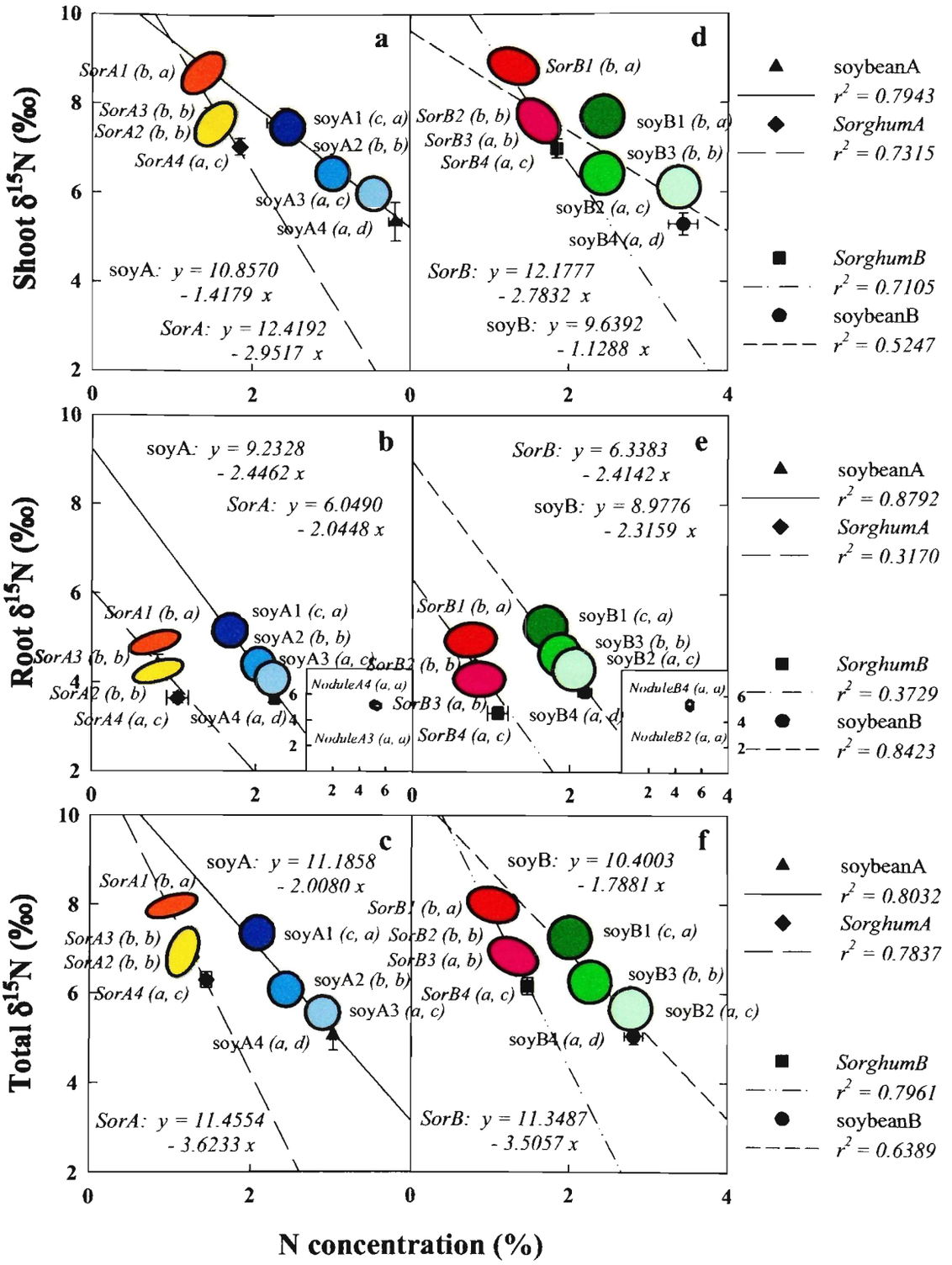

Figure 4.24. Relationships between N concentration and $\delta^{15}N$ value of soybeanA (N-donor) and *SorghumA* (N-receiver) (a-c); and *SorghumB* (N-donor) and soybeanB (N-receiver) (d-f). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x, y* parameters, respectively].

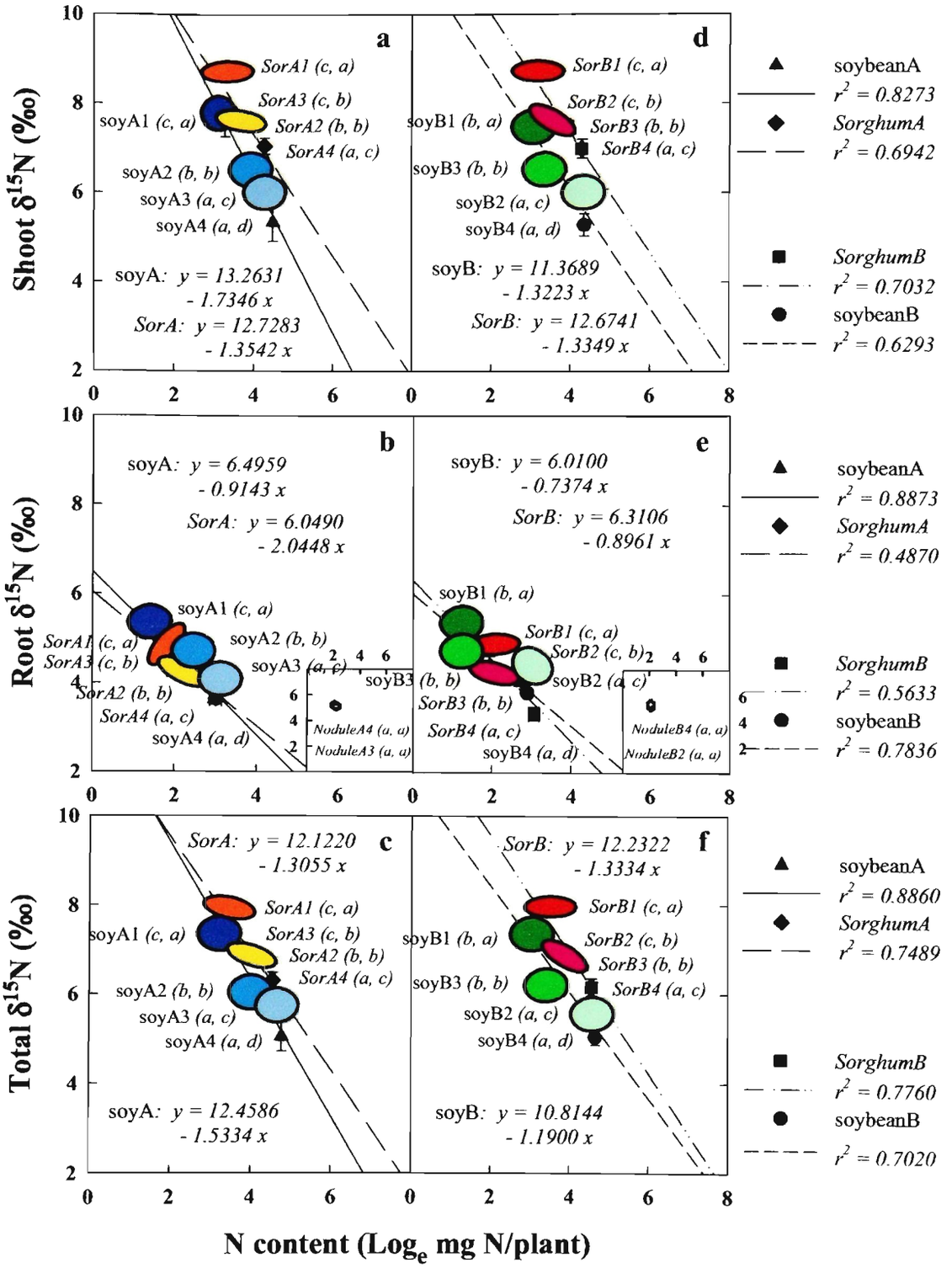

Figure 4.25. Relationships between N content and $\delta^{15}N$ value of soybeanA (N-donor) and *SorghumA* (N-receiver) (a-c); and *SorghumB* (N-donor) and soybeanB (N-receiver) (d-f). [Means ± SE, n = 9; different letter (*a, a*) signifies difference at P = 0.05 for *x*, *y* parameters, respectively].

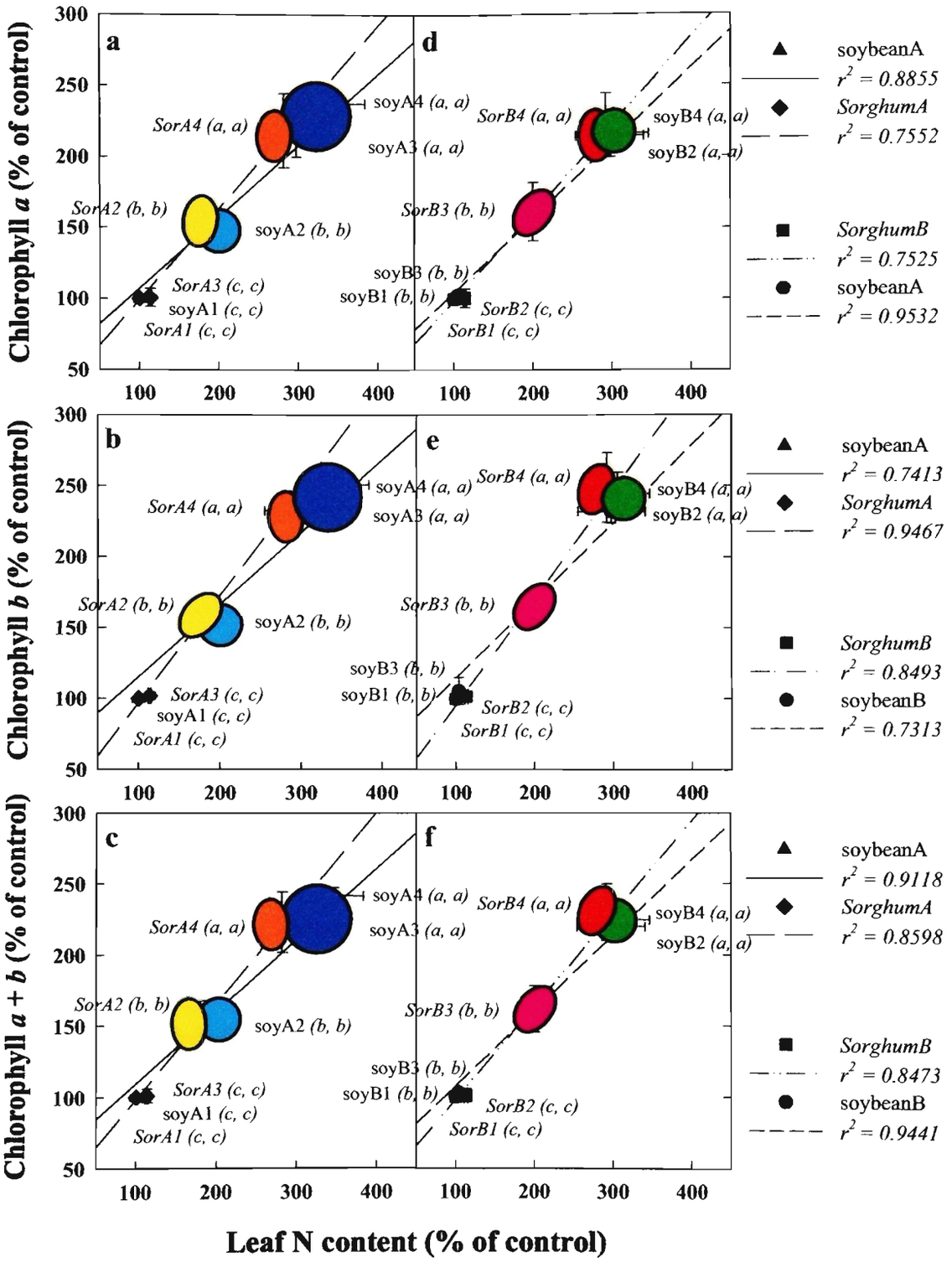

Figure 4.26. Relationships between leaf N content and chlorophyll *a*, *b* and *a* + *b* of soybeanA (N-donor) and *SorghumA* (N-receiver) (a-c); and *SorghumB* (N-donor) and soybeanB (N-receiver) (d-f). [Means ± SE, n = 9; different letter (*a*, *a*) signifies difference at P = 0.05 for *x*, *y* parameters, respectively].

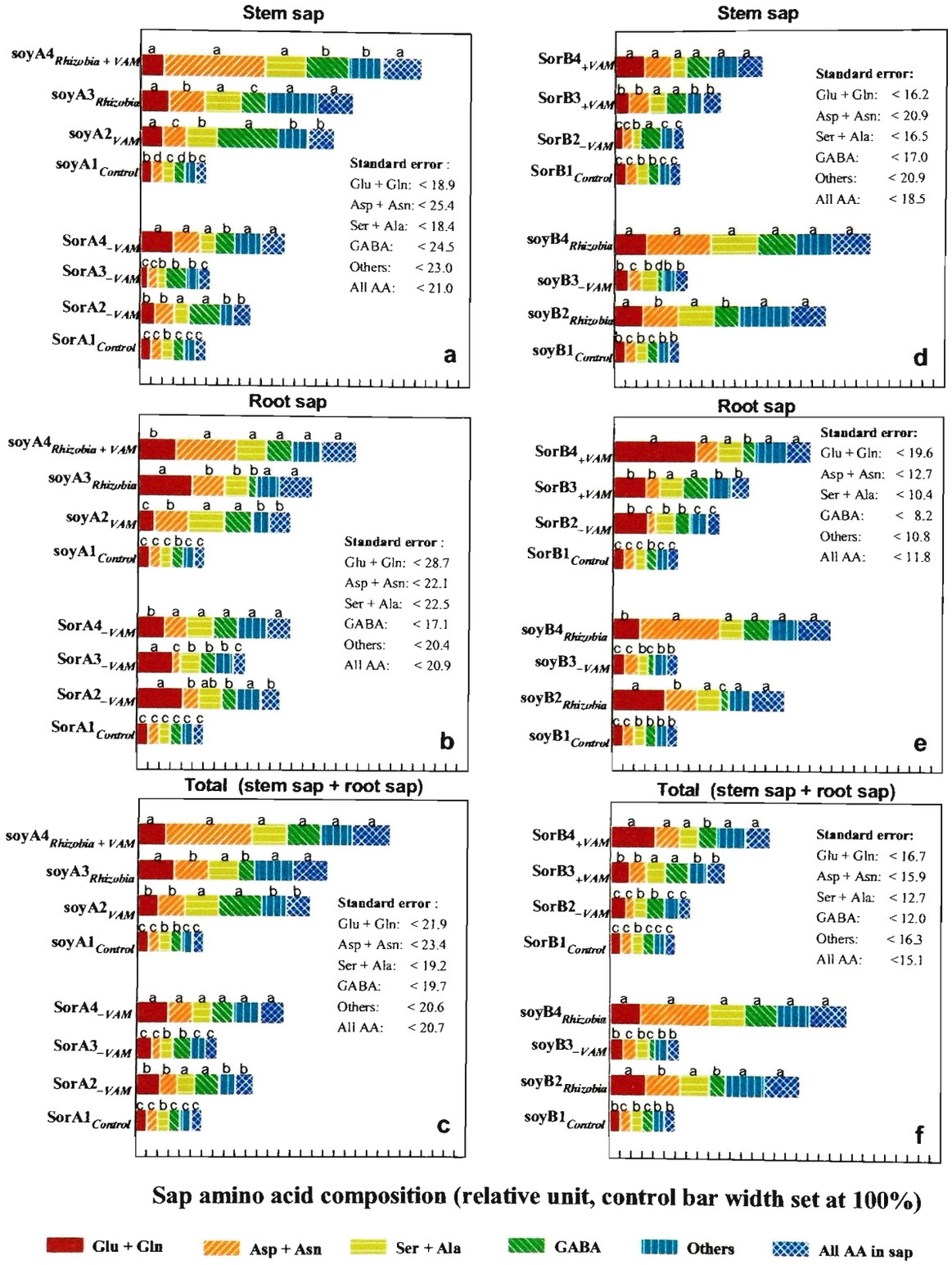

Figure 4.27. Amino acid composition of soybeanA (N-donor) and *SorghumA* (N-receiver) (a-c); and *SorghumB* (N-donor) and soybeanB (N-receiver) (d-f). N-minus experiment. [Means ± SE, n = 9; different letter (*a*, *a*) signifies difference at P = 0.05 for the same amino acid, respectively].

4.3.2.2.7 *Nitrogen transfer between soybean and Sorghum*

Table 4.11. N-transfer between soybeanA (N-donor) and *SorghumA* (N-receiver) (A), and *SorghumB* (N-donor) and soybeanB (N-receiver) (B) after 12 weeks growth in the glasshouse. Both N-donor and N-receiver were continuously fed with ^{14}N nutrition for 3 weeks for plant establishment from sowing, and then without N-supply for another 9 weeks till harvesting [Values are Means, n = 9, different letter (a, b) signifies difference at P = 0.05].

Treatments	N-transfer											
	δ^{15}N (‰)*			% $N_{transfer}$			$N_{transfer}$ (mg/plant)			% NDFT		
	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total
ExperimentA: N-transfer from soybean (N-donor) to *Sorghum* (N-receiver)												
soyA4→*SorA4*	7.05c	3.64c	6.30c	19.61a	25.43a	22.52a	17.71a	4.98a	22.69a	24.65a	24.57a	24.61a
soyB2→*SorA2*	7.55b	4.22b	6.80b	13.99b	13.79b	13.89b	7.06b	1.45b	8.51b	15.26b	11.20b	13.23b
soyB1→*SorA1*	8.78a	4.89a	7.99a	—	—	—	—	—	—	—	—	—
ExperimentB: N-transfer from *Sorghum* (N-donor) to soybean (N-receiver)												
***SorB4*→soyB4**	5.26c	3.78c	5.09c	29.84a	27.09a	28.47a	22.19a	5.93a	28.12a	28.29a	32.49a	30.39a
***SorB3*→soyB3**	6.45b	4.66b	6.17b	14.17b	10.02b	12.06b	7.26b	1.46b	8.66b	29.10a	29.27a	29.19a
***SorB1*→soyB1**	7.55a	5.18a	7.22a	—	—	—	—	—	—	—	—	—

* δ^{15}N value of N-receiver; shoot and root δ^{15}N value = 2.35‰ and 1.21‰ for nodulated soybean cultivated in an N-free medium.

Table 4.11 indicated a two-way mycorrhiza-mediated N-transfer occurred between soybean and *Sorghum*. Except for the %NDFT, both the % N-transfer and the amount of N-transferred were in the same range in the sole mycorrhizal pairs, irrespective of the direction of N-transfer. However, these two parameters, as well as the %NDFT, were higher in the direction from *Sorghum* to soybean in the nodulated mycorrhizal pairs.

4.4 ^{15}N Natural Abundance Studies Show that More N Moves to N_2-fixing Plants

4.4.1 Similar N movement in pure *Eucalyptus*/*Eucalyptus* or *Casuarina*/*Casuarina* pairs

The results in Tables 4.12 and 4.13 demonstrated, for the first time, that N-transfer occurred two-way or bidirectionally within the same species of *Casuarina* or *Eucalyptus*. The data suggested a similar N-transfer trend within the same species, either in the sole mycorrhizal or the dual nodulated mycorrhizal pairs. Also, there was

no obvious net gain or loss with regard to the direction of N-transfer in the sole mycorrhizal pairs of eucalypts or casuarinas, and the *Casuarina* pairs in which N-donor and N-receiver were nodulated and mycorrhizal. However, the nodulated mycorrhizal *Casuarina* plants, rather than the sole mycorrhizal ones, did gain more N (Table 4.13).

Table 4.12. N-transfer in pure system of *Eucalyptus* or *Casuarina* pairs

Experiment	N-transfer (%)				N-transferred (mg/plant)				NDFT (%)			
	Sole myc/pairs		Dual fr/m pairs		Sole myc/pairs		Dual fr/m pairs		Sole myc/pairs		Dual fr/m pairs	
	▲A_m➡▼B_m➡ $B_{(nm)m}$	$A_{(nm)m}$➡B_m	$A_{fr/(nm)m}$ ➡B_m	B_m➡ $A_{fr/(nm)m}$	A_m➡ $B_{(nm)m}$	B_m➡ $A_{(nm)m}$	$A_{fr/(nm)m}$ ➡B_m	B_m➡ $A_{fr/(nm)m}$	A_m➡ $B_{(nm)m}$	B_m➡ $A_{(nm)m}$	$A_{fr/(nm)m}$ ➡B_m	B_m➡ $A_{fr/(nm)m}$
+N/ECM*	9.8*a*	10.0*a*	na	na	78.3*a*	75.2*a*	na	na	9.9*a*	10.8*a*	na	na
+N/ECM**	18.1*a*	18.8*a*	29.0*b*	31.1*b*	282.6*b*	278.3*b*	419.9*a*	471.9*a*	20.0*b*	19.4*b*	29.0*a*	31.4*a*
	A_m➡ $B_{(nm)m}$	B_m➡ $A_{(nm)m}$	$A_{fr/m}$➡ $B_{fr/(nm)m}$	$B_{fr/(nm)m}$ ➡$A_{fr/m}$	A_m➡ $B_{(nm)m}$	B_m➡ $A_{(nm)m}$	$A_{fr/m}$➡ $B_{fr/(nm)m}$	$B_{fr/(nm)m}$ ➡$A_{fr/m}$	A_m➡ $B_{(nm)m}$	B_m➡ $A_{(nm)m}$	$A_{fr/m}$➡ $B_{fr/(nm)m}$	$B_{fr/(nm)m}$ ➡$A_{fr/m}$
+N/ECM**	18.1*a*	18.8*a*	31.7*b*	32.6*b*	282.6*b*	278.3*b*	1162.3*a*	1154.8*a*	20.0*b*	19.4*b*	33.6*a*	33.1*a*

▲A: *CasuarinaA, CasuarinaX* or *EucalyptusA,* ▼B: *CasuarinaB, CasuarinaY* or *EucalyptusB;* plants were 12-months-old [12 or 11 months nutrition with 4.0mM $(^{14}NH_4)_2SO_4$ to N-donor or N-receiver]. M or myc: mycorrhizal infected; (nm)m: initially non-mycorrhizal but mycorrhizal colonised through its partner during experiment; fr: *Frankia* nodulated; fr/(nm)m: initially non-mycorrhizal *Frankia* infected but mycorrhizal colonised through its partner during experiment; na: not available. Values are means, n = 9 for *Casuarina* and *Eucalyptus*, respectively, different letter (*a, b*) signifies difference at P = 0.05 for the individual parameter in the same row. **Eucalyptus* pairs, ***Casuarina* pairs.

Table 4.13. Bidirectional and net N-transfer in pure system of *Eucalyptus* or *Casuarina* pairs

Experiment	Bidirectional transfer (mg)			Net transfer (mg)		
	Sole myc-pairs	Dual fr/m pairs		Sole myc-pairs	Dual fr/m pairs	
	▲A_m➡$B_{(nm)m}$ + $B_{(nm)m}$➡A_m	$A_{fr/(nm)m}$➡B_m + B_m➡$A_{fr/(nm)m}$	$A_{fr/m}$➡$B_{fr(nm)m}$ − $B_{fr(nm)m}$➡$A_{fr/m}$	A_m➡$B_{(nm)m}$ $B_{(nm)m}$➡A_m	$A_{fr/(nm)m}$➡B_m − B_m➡$A_{fr/(nm)m}$	$A_{fr/m}$➡$B_{fr(nm)m}$ − $B_{fr(nm)m}$➡$A_{fr/m}$
+N/ECM*	153.5	na	na	+3.1	na	na
+N/ECM**	560.9*c*	891.8*b*	2342.8*a*	+4.3*b*	−52.0*a*	+7.5*b*

▲Abbreviations also see Table 4.12.

4.4.2 More N moves to *Casuarina* or soybean in *Eucalyptus*/*Casuarina* or *Sorghum*/soybean pairs

The results in Table 4.14 showed that two-way N-transfer occurred between ECM *Casuarina* and *Eucalyptus* pairs, and between AM soybean and *Sorghum* pairs, with or without the external N-supplement. The sole mycorrhizal pairs showed similar N-transfer trends and very similar amounts of N-transfer irrespective of the N-flux direction. The dual nodulated mycorrhizal pairs transferred greater amounts of N than the sole mycorrhizal pairs. Mycorrhizal *Eucalyptus* or *Sorghum* delivered more N to the nodulated mycorrhizal *Casuarina* or soybean, indicating a net N gain from their non-N_2-fixing partners (Table 4.15). In the N-minus experiments, *Casuarina* gained some external N from the atmosphere, and *Sorghum* obtained extra N from the mixed AM inoculum containing some external N. In addition, the total amount of N-transferred between *Casuarina* and *Eucalyptus* in the N-plus experiment was much higher than that in the N-minus experiment.

Table 4.14. Nitrogen transfer in *Casuarina*/*Eucalyptus* or soybean/*Sorghum* pairs

	N-transfer (%)				N-transferred (mg/plant)				NDFT (%)			
Experiment	Sole myc-pairs		Dual fr/m pairs		Sole myc-pairs		Dual fr/m pairs		Sole myc-pairs		Dual fr/m pairs	
	$^{\blacktriangle}A_m\rightarrow$ $B_{(nm)m}$	$^{\blacktriangledown}B_m\rightarrow$ $A_{(nm)m}$	$A_{nix/m}\rightarrow$ $B_{(nm)m}$	$B_m\rightarrow$ $A_{nix/(nm)m}$	$A_m\rightarrow$ $B_{(nm)m}$	$B_m\rightarrow$ $A_{(nm)m}$	$A_{nix/m}\rightarrow$ $B_{(nm)m}$	$B_m\rightarrow$ $A_{nix/(nm)m}$	$A_m\rightarrow$ $B_{nm)m}$	$B_m\rightarrow$ $A_{(nm)m}$	$A_{nix/m}\rightarrow$ $B_{(nm)m}$	$B_m\rightarrow$ $A_{nix/(nm)m}$
+N/ECM*	10.4*b*	11.0*b*	24.0*a*	26.7*a*	83.9*c*	88.8*c*	200.6*b*	353.7*a*	11.8*b*	7.4*c*	33.6*a*	11.4*b*
–N/ECM**	13.0*b*	11.9*b*	22.1*a*	25.1*a*	5.9*c*	5.3*c*	16.2*b*	19.8*a*	15.2*b*	3.8*c*	28.5*a*	10.7*b*
–N/AM***	13.9*c*	12.1*c*	22.5*b*	28.5*a*	8.5*c*	9.0*c*	22.6*b*	28.0*a*	13.2*b*	29.2*a*	24.6*a*	30.4*a*

$^{\blacktriangle}$A: *Casuarina* or soybean; $^{\blacktriangledown}$B: *Eucalyptus* or *Sorghum*; nix: *Frankia* or *Rhizobium* nodulated; other abbreviations also see Table 4.12. Values are means, n = 9, different letter (*a, b*) signifies difference at P = 0.05 for the individual parameter in the same row.

*12-months-old seedlings [12 or 11 months nutrition with 4.0mM $(^{14}NH_4)_2SO_4$ to N-donor or N-receiver, respectively]; **6-months-old seedlings [6 weeks nutrition with 4.0mM $(^{14}NH_4)_2SO_4$ to both N-donor and N-receiver, then without N-supply for another 18 weeks]; ***3-months-old seedlings [3 weeks nutrition with 4.0mM $(^{14}NH_4)_2SO_4$ to both N-donor and N-receiver, then without N-supply for another 9 weeks].

Table 4.15. Bidirectional and net nitrogen transfer in *Casuarina/Eucalyptus* or soybean/*Sorghum* pairs

	Bidirectional transfer (mg)		Net transfer (mg)	
Experiment	Sole myc-pairs*	Dual nix/m pairs	Sole myc-pairs	Dual nix/m pairs
	$A_m \rightarrow B_{(nm)m}$ + $B_m \rightarrow A_{(nm)m}$	$A_{nix/m} \rightarrow B_{(nm)m}$ + $B_m \rightarrow A_{nix/(nm)m}$	$A_m \rightarrow B_{(nm)m}$ − $B_m \rightarrow A_{(nm)m}$	$A_{nix/m} \rightarrow B_{(nm)m}$ − $B_m \rightarrow A_{nix/(nm)m}$
+N/ECM	172.7*b*	554.3*a*	–4.9*b*	–153.1*a*
–N/ECM	11.2*b*	36.1*a*	+0.5*b*	–3.8*a*
–N/AM	17.2*b*	50.8*a*	–0.2*b*	–5.4*a*

*Abbreviations also see Table 4.14.

4.5 Validity of N-transfer from ^{15}N Natural Abundance

So far, the methodologies for investigating nutrient transfer between plants, via indirect soil pathways or direct mycorrhiza-mediated pathways, mostly make use of tracers such as ^{14}C, ^{32}P and ^{15}N (though ^{15}N is not a radioactive tracer!). These isotopes were added to the growth media or injected into the plant itself, and analysis of these tracers was then used to monitor the movement of them within or between plants. There is only one report that tried to use δ^{15}N for investigating N-transfer between non-mycorrhizal N_2-fixing and non-N_2-fixing trees (Binkley *et al.*, 1985). They reported a non-consistent, significantly different variation of δ^{15}N values, between the N_2-fixing alders and the non-N_2-fixing Douglas firs, suggesting that δ^{15}N natural abundance analysis is not promising for evaluating N-transfer between plants.

Normally, the δ^{15}N‰ value of an N_2-fixing plant that actively fixes atmospheric dinitrogen will be close to the standard zero of the air (Mariotti, 1983). On the one hand, Shearer and Kohl (1986) pointed out that the lighter ^{14}N is usually kinetically favoured over the heavier ^{15}N by plants in both biotic and abiotic reactions, resulting in an ^{15}N isotope enrichment in the remaining N-source. On the other hand, studies have also shown that host plants and mycorrhizal associates differ in their δ^{15}N values by as much as ~8‰ (Handley and Scrimgeour, 1997; Högberg, 1997). It has

been hypothesised that such a $\delta^{15}N$ difference is due to the discrimination against ^{15}N when N is translocated from the mycorrhizal fungus to the host plant while the former generally retains more ^{15}N atoms than is present in the N-source (Gebauer and Taylor, 1999). More recently, Emmerton *et al.* (2001a, b) found that ^{15}N abundance of mycorrhizal fungi could be significantly altered during N uptake and metabolism, and that plant ^{15}N abundance always differed from the N-sources and was influenced by mycorrhizae as well, irrespective of the inorganic or organic N source used. To some extent, any difference in $\delta^{15}N$ value between source and sink should thus reflect the N movement or shift associated with the specific N-source. As a consequence, it seems plausible that $\delta^{15}N$ or the $\delta^{15}N$ difference between source and sink, can trace mycorrhiza-mediated N-transfer between plants.

The N-transfer data generated from the ^{15}N labeling experiment (Table 3.6) have shown results consistent with many previous studies. Table 4.16 gives a comparison of N-transfer in the 12-months-old plants from the analyses of ^{15}N labeling and $\delta^{15}N$ natural abundance. Generally, the comparison shows a similar N-transfer trend in *Casuarina/Eucalyptus* pairs with these two different ^{15}N analyses. For example, the %N-transfer and %NDFT in the sole mycorrhizal pairs ranged between 2-14% and 4-10% in the ^{15}N labeling experiments, while they were between 10-11% and 7-12% in the $\delta^{15}N$ the natural abundance experiments. However, in the dual nodulated mycorrhizal pairs, they were between 9-41% and 22-33% in the ^{15}N labeling experiments, and 24-27% and 11-35% and in $\delta^{15}N$ natural abundance experiments.

Table 4.16. Comparison of N-transfer between ^{15}N labeling and $\delta^{15}N$natural abundance analysis

Treatments	N-transfer (%)				N-transferred (mg/plant)				NDFT (%)			
	Sole myc-pairs		Dual fr/m pairs		Sole myc-pairs		Dual fr/m pairs		Sole myc-pairs		Dual fr/m pairs	
	▲C_m➡ $E_{(nm)m}$	E_m➡ $C_{(nm)m}$	$C_{fr/m}$➡ $E_{(nm)m}$	E_m➡ $C_{fr/(nm)m}$	C_m➡ $E_{(nm)m}$	E_m➡ $C_{(nm)m}$	$C_{fr/m}$➡ $E_{(nm)m}$	E_m➡ $C_{fr/(nm)m}$	C_m➡ $E_{(nm)m}$	E_m➡ $C_{(nm)m}$	$C_{fr/m}$➡ $E_{(nm)m}$	E_m➡ $C_{fr/(nm)m}$
12-months-old seedlings (11 months nutrition with 4.0mM ^{14}N to plants, then 1 month 4.0mM ^{15}N to N-donor only)												
$^{15}NH_4NO_3$	2.9$_{(b, c)}$	14.4$_{(a, b)}$	12.8$_{(b, b)}$	40.6$_{(a, a)}$	8.0$_{(b, c)}$	11.4$_{(b, c)}$	72.2$_{(b, b)}$	169.0$_{(b, a)}$	9.6$_{(a, b)}$	4.7$_{(a, b)}$	33.0$_{(a, a)}$	30.0$_{(a, a)}$
$NH_4{}^{15}NO_3$	2.1$_{(b, c)}$	8.6$_{(a, b)}$	8.8$_{(b, b)}$	35.0$_{(a, a)}$	7.9$_{(b, c)}$	10.2$_{(b, c)}$	46.4$_{(c, b)}$	191.6$_{(b, a)}$	6.7$_{(a, b)}$	3.8$_{(a, b)}$	22.0$_{(a, a)}$	22.4$_{(a, a)}$
12-months-old seedlings (12 or 11 months nutrition with 4.0mM ^{14}N to N-donor or N-receiver, respectively)												
$(^{14}NH_4)_2SO_4$	10.4$_{(a, b)}$	11.0$_{(a, b)}$	24.0$_{(a, a)}$	26.7$_{(b, a)}$	83.9$_{(a, b)}$	88.8$_{(a, b)}$	200.6$_{(a, a)}$	353.7$_{(a, a)}$	11.8$_{(a, b)}$	7.4$_{(a, c)}$	33.6$_{(a, a)}$	11.4$_{(b, a)}$

▲C: *Casuarina*; E: *Eucalyptus*, other abbreviations also see Table 4.12.

As for the amounts of N-transferred, it seems that the $\delta^{15}N$ natural abundance analysis yields much higher amounts (Table 4.17). However, the biomass and N content in both species nearly doubled in the $\delta^{15}N$ natural abundance experiments, probably due to different external N fertilisation to the growth media, and different transplanting and harvest dates. More importantly, there was only one ^{15}N atom present in the two N-sources, $^{15}NH_4{}^{14}NO_3$ and $^{14}NH_4{}^{15}NO_3^-$, in the ^{15}N labeling experiments, while a two ^{14}N atom source, $(^{14}NH_4)_2SO_4$, was used for cultivating the plants in the $\delta^{15}N$ natural abundance experiments. As a consequence, the amounts of N-transferred in the ^{15}N labeling experiments should be doubled if there was a similar ^{14}N transfer, concurrent with the ^{15}N transfer, between plants. That is to say, the amount of N-transferred was similar when these two techniques were used. Thus the $\delta^{15}N$ natural abundance analysis is a worthwhile tool, for further N-transfer investigation.

Table 4.17. Comparison of biomass and nitrogen content in 12-months-old *Casuarina* and *Eucalyptus* seedlings

	^{15}N labeling Experiments				$\delta^{15}N$ Experiments			
Plants	*Casuarina*		*Eucalyptus*		*Casuarina*		*Eucalyptus*	
	C_m	$C_{fr/m}$	E_m	E_m	C_m	$C_{fr/m}$	E_m	E_m
Treatments	+	+	+	+	+	+	+	+
	$C_{(nm)/m}$	$C_{fr/(nm)/m}$	$E_{(nm)/m}$	E_m	$C_{(nm)/m}$	$C_{fr/(nm)/m}$	$E_{(nm)/m}$	E_m
Biomass	64*	85	41	48	119	186	69	74
Biomass	65**	87	42	49				
Nitrogen	742***	1446	254	398	1210	3174	810	885
Nitrogen	755****	1406	295	414				

*g/plant, labeled with $^{15}NH_4NO_3$ (hereafter in the same row); **g/plant, labeled with $NH_4{}^{15}NO_3$; ***mg/plant, labeled with $^{15}NH_4NO_3$; ****mg/plant, labeled with $NH_4{}^{15}NO_3$; abbreviations also see Table 4.12.

CHAPTER 5 GENERAL DISCUSSION

The collective evidence from studies of one-way N-transfer from the N_2-fixing plants to the non-N_2-fixing plants, through either common AM or ECM networks, indicates that the direction of N-flow is determined by the source-sink relationship, i.e., N flows from the N-rich N_2-fixing plants (N-donor, source) to the N-poor non-N_2-fixing ones (N-receiver, sink). Discussed below are the key findings in this thesis:

1. The experiments conducted in this study provide new evidence of mycorrhiza-mediated one-way N-flow from the N_2-fixing plants to the non-N_2-fixing ones;
2. An unusual direction of mycorrhiza-mediated N-flow from the N-poor non-N_2-fixing plants to the N-rich N_2-fixing ones;
3. Two-way mycorrhiza-mediated N-flow between pure systems of either N_2-fixing plants or non-N_2-fixing plants.

As a consequence, it is proposed that two-way (bi-directional) mycorrhiza-mediated N-transfer can occur between any mycorrhizal plant species, irrespective of their N_2-fixation abilities, although the N_2-fixation activity may influence the intensity of such a two-way N-transfer.

5.1 Total Mycorrhizal Infection

Mycorrhizae are three-way symbioses involving plants, fungi and soils (Brundrett *et al.*, 1996; Smith and Read, 1997). In this way, plants and fungi collaborate mutualistically to form mycorrhization, the fungi gaining 10-20% of the net photosynthate from plants and pumping soil nutrients into the plants in return. About 90% of the known plants form mycorrhizal associations in nearly every terrestrial habitat in the world. Two-thirds of these plants are symbiotic with AM fungi while most of the rest associate with ECM fungi (Trappe, 1987; Smith and Read, 1997). A variety of methods have been used to examine and quantify root mycorrhizal infection (Brundrett *et al.*, 1996). Of those, the gridline intersection method is most commonly used, and the total mycorrhizal infection is presented as the proportion (%) of root length occupied or colonised by the fungus. Plants obtain an optimal maximum level of root infection in glasshouse and/or in field environments, depending on plant species, soil and climatic conditions and the fungus species.

Australian scientists started mycorrhizal research in *Eucalyptus* in the 1920's (Chilvers and Pryor, 1965). *Eucalyptus* is capable of forming both ECM and AM (rarely in older trees) associations, even on the same root system (Chilvers *et al.*, 1987; Boudarga and Lapeyrie, 1990; Brundrett and Abbott, 1991; Bellei, 1992; Brundrett *et al.*, 1996; May and Simpson, 1997; Chen *et al.*, 2000). A range of 10-60% of root length colonised by a variety of ECM fungi in *Eucalyptus*, including some *Pisolithus* spp., was generally found either under glasshouse pot cultivation or in natural ecosystems (Malajczuk *et al.*, 1981; Lapeyrie and Chilvers, 1985; Chilvers *et al.*, 1987; Boudarga and Lapeyrie, 1990; Brundrett and Abbott, 1991; Reddell and Mines, 1992; May and Simpson, 1997; Reddy and Satyanarayana, 1998; Launonen *et al.*, 1999; Chen *et al.*, 2000; Howard *et al.*, 2000; Mason *et al.*, 2000a, b, c; Sharma and Adholeya, 2000; Sastry *et al.*, 2000).

Casuarina is also colonised by both ECM and AM fungi and generally has 10-40% of ECM and 10-50% of AM root infection, respectively (Gauthier *et al.*, 1983; Gardner, 1986; Theodorou and Reddell, 1991; Sempavalan *et al.*, 1995; Subbarao and Rodriguez-Barrueco, 1995; Reddell *et al.*, 1997a; Osundina, 1998; Singh *et al.*, 1998; Mark *et al.*, 1999; Wheeler *et al.*, 2000). Tripartite relations between ECM and/or AM fungi and the N_2-fixing bacterium *Frankia* are common in *Casuarina* plants as well (Gauthier *et al.*, 1983; Gardner, 1986; Theodorou and Reddell, 1991; Subbarao and Rodriguez-Barrueco, 1995; Reddell *et al.*, 1997a). However, root colonisation was statistically the same between the dual *Frankia*/mycorrhizal and the sole mycorrhizal casuarinas (Gauthier *et al.*, 1983; Gardner, 1986).

Cereals are colonised by AM fungi only. Root mycorrhizal infection in cereals varies greatly with environmental conditions. Up to 70% of root colonisation has been observed in soybean (Bethlenfalvay *et al.*, 1991; Hamel and Smith, 1991; Hamel *et al.*, 1991; Sieverding, 1991; Hamel and Smith, 1992; Martin *et al.*, 1995; Bethlenfalvay *et al.*, 1997, 1999; Khalil *et al.*, 1999; McGonigle *et al.*, 1999; Mujica *et al.*, 1999; Sanginga *et al.*, 1999; Ezawa *et al.*, 2000; Shrihari *et al.*, 2000; Auge *et al.*, 2001; Kelly *et al.*, 2001) and *Sorghum* (Graham *et al.*, 1982; Ocampo, 1986; Raju *et al.*, 1990; Hawkins and George, 1997; Brundrett M.C. *et al.*, 1999; Caris *et al.*, 1999; Godeas *et al.*, 1999; Abdel-Fattah and Mohamedin, 2000; Bagayoko *et al.*, 2000a, b; Fonseca *et al.*, 2001). Tripartite symbioses between AM fungi and the N_2-

fixing bacterium *Rhizobium* were also reported in leguminous plants including soybean. In general, AM inoculation always had a positive effect on the nodulation of legumes (Cluett and Boucher, 1983). However, the mycorrhization level did not differ significantly between the nodulated mycorrhizal and the non-nodulated mycorrhizal soybeans (Badr El-din and Moawad, 1988; Bethlenfalvay *et al.*, 1991; Hamel *et al.*, 1991; Hamel and Smith, 1992; Martin *et al.*, 1995). More recently, there have been hypotheses that the *Rhizobium*-legume symbiosis may have evolved from the AM association (Gianinazzi-Pearson, 1996; Gough *et al.*, 1999; see Bloom and Holbrook, 2001), and that mycorrhizal formation and nodulation in pea, broad bean and lucerne are regulated by similar or the same processes (Hirsch, 1992; Gianinazzi-Pearson, 1996; Resendes *et al.*, 2001).

The capacity to form mycorrhizal associations is generally recognised to be essential for plant seedlings to become established in terrestrial habitats where inocula of mycorrhizal fungi are limited or lacking, especially in poor nutrient environments (Harley and Smith, 1983; Allen, 1991, 1992; Brundrett *et al.*, 1996; Smith and Read, 1997). Nowadays, mycorrhizal inocula are routinely applied in a great deal of agricultural and forestry practices (Brundrett *et al.*, 1996; Smith and Read, 1997; Mukerji *et al.*, 2000). In both the pure and paired experiments described in Chapters 3 and 4, root mycorrhizal infection was lowest (40%) in *Casuarina* and highest (80%) in *Sorghum*. The ECM infections in *Casuarina* and *Eucalyptus*, and the AM infections in soybean and *Sorghum* were generally higher than previously reported by other researchers, because of procedures for developing mycorrhizal seedlings in the well-controlled aseptical room conditions, and in the favourable, controlled glasshouse environment for plant growth.

5.2 Common Mycorrhizal Networks (CMNs) between Plants

Plants usually grow naturally close together, in a single-species population, but also in multiple-species communities. The majority of plants, especially grasses and trees, hide almost half their biomass in an enormous tangle of roots in the below-ground soils. Mycorrhizal fungi are ubiquitous components of most soil ecosystems. They grow through the soil, colonise the roots of various plants and are capable of forming

links between plant species (Newman, 1988; Newman *et al.*, 1992; 1994). Because of little host specificity in colonisation (Harley and Smith, 1983; Smith and Read, 1997), plant roots are linked by a common mycorrhizal network (CMN) with either AM or ECM fungi, forming 'the wood-wide-web', a term coined by the prestigious scientific journal *Nature* (see the cover of Volume 388, 7 August 1997). These, in turn, are usually woven into an even bigger tangle of fungi and roots (Newman, 1988; Perry *et al.*, 1989; Read, 1992; 1997).

Mycorrhizal links between plants have been shown by direct observation from transparent microcosms (Heap and Newman, 1980a, b; Francis and Read, 1984; Finlay and Read, 1986a, b; Newman, 1988; Newman *et al.*, 1992; 1994) and by isotope autoradiography (Hirrel and Gerdemann, 1979; Chiariello *et al.*, 1982; Francis and Read, 1984; Read *et al.*, 1985; Finlay and Read, 1986a, b; McKendrick *et al.*, 2000; Wu *et al.*, 2000). There is also indirect evidence that plants from the same population are able to share a more efficient hyphal network (Ronsheim and Anderson, 2001). Groups of plant species joined together in this way have been known as functional guilds (Perry *et al.*, 1989), which facilitate nutrient uptake and translocation (Read, 1997). Nutrients such as C (Bjorkman, 1960; Reid and Woods, 1969; Hirrel and Gerdemann, 1979; Brownlee *et al.*, 1983; Francis and Read, 1984; Read *et al.*, 1985; Filay and Read, 1986a; Grime *et al.*, 1987; Duddridge *et al.*, 1988; Martins, 1992, 1993; Waters and Borowicz, 1994; Ek *et al.*, 1996; Watkins *et al.*, 1996; Graves *et al.*, 1997; Simard *et al.*, 1997a, c; Fitter *et al.*, 1998; Wu *et al.*, 2001; Lerat *et al.*, 2002), N (van Kessel *et al.*, 1985; Haystead *et al.*, 1988; Eissenstat, 1990; Bethlenfalvay *et al.*, 1991; Frey and Schuepp, 1992, 1993; Arnebrant *et al.*, 1993; Johansen *et al.*, 1993a, b; Ikram *et al.*, 1994; Ekblad and Huss-Danell, 1995; Ek *et al.*, 1996; Johansen and Jensen, 1996; Martins and Cruz, 1998) and P (Heap and Newman, 1980b; Chiariello *et al.*, 1982; Whittingham and Read, 1982; Finlay and Read, 1986b; Newman and Ritz, 1986; Eason *et al.*, 1991; Newman and Eason, 1993; Johansen and Jensen, 1996; Martins and Read, 1996; Tuffen *et al.*; 2002) have been found to move through the CMNs, from plant to plant. Also, such networks "would be expected to reduce dominance of aggressive species, so promoting coexistence and greater biodiversity" (Read, 1997) if it would result in an equalisation of resource availability within the plant community.

We investigated the formation of CMNs between Australian native *Casuarina* and *Eucalyptus*, or within the same species of *Casuarina* or *Eucalyptus*, with the ECM fungus *Pisolithus tinctorius*, and between field crops soybean and *Sorghum* with the AM fungus *Glomus mosseae*. The mycorrhization results in Chapters 3 and 4 showed that all the initially non-mycorrhizal seedlings were colonised after the experiments through their mycorrhizal partners, indicating that common hyphal links had been established between the same or the different species. Together with direct visual observations by light and Environmental Scanning Electron Microscopy, and root mycorrhizal infections as high as 40% in *Casuarina* and 70% in *Eucalyptus*, it can be concluded that a CMN between these two predominant Australian native trees has been established. Root length colonisation as high as 75% in soybean and 80% in *Sorghum* also indicated a common AM network between them. As far as we know, these are the first reports of a common ECM network between *Casuarina* and *Eucalyptus*, within either *Casuarina* or *Eucalyptus*, and a common AM network between soybean and *Sorghum* in pot cultivation conditions. More importantly, these four newly discovered CMNs have the same interspecific and intraspecific nutrient exchange capacity as a number of other plants for N.

Read *et al.* (1985) suggested that transfer of nutrients from established plants to seedlings might be a crucial factor enabling young plants to survive in nutrient-limited situations. Smith and Read (1997) placed stress on "mycorrhizal symbiotic association as a central strategy for improved nutrient capture from soil in most plants". Read (1997) further speculated about the potential ecological importance of C transfer through a common ectomycorrhizal network – "mycorrhizal linkage would reduce dominance of aggressive species, so promoting coexistence and greater biodiversity". As Read (1997) has already suggested, "we should place less emphasis on competition between plants and more on the distribution of resources within the community". *Casuarina* and *Eucalyptus* grow naturally on the Australian continent (Attiwill and Adams, 1996; Williams and Brooker, 1997) and are widely planted as pure or mixed stands around the world (Eldridge *et al.*, 1993; Williams and Woinarski, 1997; Doughty, 2000). Soybean is widely intercropped with maize or *Sorghum* in agriculture throughout the world (Fageria *et al.*, 1995; Chalk 1998). Therefore, the ability of these two pairs to form CMNs in open field situations could have important impacts on both agricultural and natural ecosystems based on these

species. Such CMNs could affect the ability of seedlings to establish, influence source competition between established plants, accelerate the rate of nutrient cycling between them, and access nutrients from dying roots – a common phenomenon in mixed agricultural and natural systems. The growth performance of living trees, cereals or grasses could also be greatly affected by CMNs. If all of these are important, our current views of resource capture and nutrient cycling in pure, intercropped and mixed plant communities have to be reassessed.

5.3 Responses of Plant Growth to Mycorrhizal Colonisation

Asai probably made the first observation of plant growth responses to mycorrhizal colonisation in 1944 (see Smith and Read, 1997). He indicated that mycorrhizal infection was important for plant growth and nodule development of a large number of legumes. Since then, many researchers have independently investigated the basis for these growth responses in several plant species, including a variety of crop plants and trees growing in different habitats. With respect to plant growth responses to mycorrhizal colonisation, as Smith and Read (1997) have pointed out, mycorrhizal seedlings were always taller, had larger root systems and greater shoot and/or root biomass, but lower root:shoot ratios. However, some reports also showed that mycorrhization did have no or even a negative effect on growth of mycorrhizal plants in conditions when photosynthesis was suppressed and/or soil-derived nutrient supplies were limited.

To date, AM and/or ECM are conclusively known to increase plant growth (shoots and roots) in many plants, including legumes and non-leguminous actinorhizal N_2-fixing plants (Barea and Azcon-Aguilar, 1983; Harley and Smith, 1983; Hayman, 1983; Gardner, 1986; Hayman, 1986; Call and Davies, 1988; Smith and Read, 1997; Sharmila *et al.*, 2000). These studies have generally emphasised the importance of mycorrhizae as a secondary agent for better nutrient uptake, especially P (Barea and Azcon-Aguilar, 1983; Gardner, 1986; Hayman, 1986; Mosse, 1986; Marschner and Dell, 1994; Smith and Read, 1997; Smith and Smith, 2001). More recently, Rao and Tak (2001) found significantly higher plant height (up to 40%) and biomass production (up to 37%) in five tree species of *Acacia ampliceps*, *A. eriopoda*, *Albizia*

lebbek, *Azadirachta indica* and *Colophospermum mopane* infected with *Glomus fasciculatum*, when compared with non-mycorrhizal controls grown in gypsum mine spoil. A significantly higher uptake of N and P was also observed in the mycorrhiza inoculated plants, particularly in the N_2-fixing *Acacia* and *Albizia*. Janos *et al.* (2001) showed a 39% higher above-ground, but no below-ground, dry matter difference in *Litchi chinensis* infected by a mixed inoculum of 14 species of Glomalean fungi when compared with the non-mycorrhizal control plants. Rogers *et al.* (2001) found that the mycorrhizal white clover (*Trifolium repens*) had a higher dry weight and N content than the non-mycorrhizal one.

Different mycorrhizal fungal species differ in their effects on plant growth. Bougher *et al.* (1990) investigated responses of plant growth with four ECM fungi *Descolea maculata* A and B, *Laccaria laccata* and *Pisolithus tinctorius*. All the mycorrhizal plants had significantly higher biomass than their non-mycorrhizal counterparts. These effects on growth were attributed to improved mineral nutrition, because most of them had a similar response to that achieved by non-mycorrhizal plants that received adequate P fertilizer. Herrmann *et al.* (1998) observed no significant corresponding biomass increase for 3-months-old *Quercus robur* L. microcuttings inoculated with either *Paxillus involutus* or *Piloderma croceum,* although both elongation and production of the lateral root system was stimulated by *P. croceum*.

Jonsson *et al.* (2001) reported the effects of eight mycorrhizal fungal species on seedling productivity of two tree species. As a general rule, mycorrhization had a significant impact on shoot and/or root weight, on shoot:root ratio of *Pinus sylvestris*, and on shoot biomass and seedling height of *Betula pendula*. Furthermore, they indicated that the effects of mycorrhizal fungal diversity on tree productivity were influenced by soil fertility. Compared to any of the eight fungal monoculture treatments, plant productivity doubled with fungal species richness (2, 4 or 8 fungi together) in low soil fertility, but no such increase in high soil fertility was seen for *B. pendula*. No apparent effects of productivity were found in low soil fertility while there were negative effects of fungal diversity on productivity for *P. sylvestris*. They concluded that effects of ECM fungal diversity on productivity were context dependent and might be positive, negative or neutral depending on nutrient status or availability. In addition, up till now studies of the effect of mycorrhization on flower

production and yield have been limited to only a few plant species (Bethlenfalvay *et al.*, 1997; Poulton *et al.*, 2001; Wilson *et al.*, 2001).

Our results also generally showed that mycorrhization had significant effects on biomass production in *Casuarina*, *Eucalyptus*, soybean and *Sorghum*. Biomass was highest when N_2-fixing plants were both nodulated and mycorrhizal. However, although root mycorrhizal infection was double in *Eucalyptus*, it had little effect on *Eucalyptus* N accumulation. Conversely, mycorrhization had a major effect on N accumulation in *Casuarina* although it had much less mycorrhizal infection. Nitrogen content reached the highest values in the nodulated mycorrhizal pairs, especially when nodulated casuarinas acted as the N-receivers that endured N nutrient stress. This was also the case for soybean and *Sorghum*. In addition, a positive and linear correlation was found in all four species when biomass was plotted against N content, that is, biomass production increased linearly with N content in both N_2-fixing plants and non-N_2-fixing ones.

5.4 Below-ground N-transfer between Plants

Virtanen *et al.* (1937) were probably the first to point out the excretion of soluble N-compounds, such as ammonium, amino acids and amides, from legume roots as a pathway of N-transfer to neighbouring cereals (Ta *et al.*, 1986, 1987; Brophy and Heichel, 1989; Paynel *et al.*, 2001). In addition to excreted N-compounds, it is now known that below-ground N-transfer can also include N derived from senesced root-cortex cells, roots and nodules (Fujita, 1992; Ledgard and Steel, 1992; Stern, 1993; Farnham and George, 1994; Chalk, 1996a, b; 1998). The deposition and decomposition of these N-containing materials from legumes have been termed rhizodeposition (Chalk, 1996a, b). As a consequence, part of the N in the rhizodeposition subsequently assimilated by adjacent non-legumes was derived from the intercropped legumes (Giller *et al.*, 1991; Jensen, 1996). The process of N deposition and uptake by an associated non-N_2-fixing plant is termed N-transfer from the N_2-fixing plant (Johansen and Jensen, 1996). The detection of ^{15}N-enrichment in the biomass of non-legumes has been considered to be evidence for such a transfer (Ta *et al.*, 1989). The general hypothesis of this one-way N-transfer is that N flows

from the N-rich legume (N-donor, source) to the N-poor non-legume (N-receiver, sink) (Bethlenfalvay *et al.*, 1991; Frey and Schuepp, 1992).

One-way below-ground flow of N from legumes to non-legumes can take place by indirect and/or direct routes (McNeill and Wood, 1990). The indirect below-ground N-transfer comes from the following materials: dead and decayed nodules and roots (Butler *et al.*, 1959; Dubach and Russelle, 1994), exudates from legume roots (Ta *et al.*, 1986), and sloughed-off root-cortex cells (Brophy and Heichel, 1989). It is the N or the N-compound in these materials that are deposited in soil and eventually absorbed by an adjacent plant. This is termed the soil pool pathway (Stern, 1993; Chalk, 1996a, b). The direct below-ground N-transfer pathway is thought to be mediated by mycorrhizal hyphae, or through common mycorrhizal networks that connect the same and different plants. Such one-way N-transfer has been demonstrated by the use of one or two fine nylon or stainless steel mesh barriers that allow the passage of hyphae but not roots, or prevent penetration of both hyphae and roots (Bethlenfalvay *et al.*, 1991; Frey and Schuepp, 1992; Arnebrant *et al.*, 1993; Johansen and Jensen, 1996). In agricultural and natural ecological communities, mycorrhiza-mediated N-transfers can be important factors influencing the performance of plants, particularly when and where the relatively immobile NH_4^+ rather than the mobile NO_3^- is the major source of plant available N.

Nitrogen could also be transferred from grasses to associated legumes, resulting in a bidirectional N-flow between the N_2-fixing plant and the non-N_2-fixing one, although such transfer was not very significant under N-limited growing conditions (Brophy *et al.*, 1987). Tomm *et al.* (1994) observed the occurrence of bidirectional N-transfer between bromegrass and lucerne. More recently, by using both direct ^{15}N leaf feeding and indirect soil ^{15}N dilution, Hogh-Jensen and Schjoerring (2000) indicated that N-transfer between N_2-fixing white/red clover (mixture) and non-N_2-fixing ryegrass was a bidirectional process. The direct ^{15}N leaf labeling indicated that N-transfer could be up to 50% from clover to ryegrass, while it was approximately 8% in the reverse direction. They also found that the indirect soil ^{15}N dilution generally led to more than a 50% underestimation of the net N-transfer compared to the direct ^{15}N leaf labeling technique. Unfortunately, none of these three reports examined the mycorrhization status of the tested plants. Johansen and Jensen (1996) did find a

two-way N-transfer between barley and pea seedlings interconnected by an AM fungus *Glomus intraradices*, although it was very low (0.3%). By contrast, as much as 15% of the pea-root N was transferred to barley when both plants were mycorrhizal and the pea shoots were removed. They concluded that the mycorrhiza-mediated N-transfer was almost insignificant in intact mycorrhizal seedlings, but significant when the root system of one of the mycorrhizal plants was decomposing. However, Rogers *et al.* (2001) did not find any N-transfer from white clover to ryegrass or *vice versa*, through hyphal links of AM fungus in agricultural soils, although mycorrhization had a positive impact on dry matter production and N yield of the clover plants. They claimed that N-transfer was independent of the fungus.

The results reported in this thesis have demonstrated that N can be translocated in either direction between the same or different species and that mycorrhizae alone or mycorrhizae/N_2-fixation together have an effect on such N flux between plants. In doing so, different treatments had specific purposes in the investigations of N-transfer between plants in the research protocol. The non-mycorrhizal controls acted as an indicator for N movement through soil pathways. The non-mycorrhizae/sole mycorrhizal pairs validated both hyphal interconnection and mycorrhiza-mediated N-transfer between species. The non-mycorrhizal nodulated N_2-fixing/mycorrhizal non-N_2-fixing plant pairs validated the participation of N_2-fixation. The nodulated mycorrhizal N_2-fixing/mycorrhizal non-N_2-fixing plant pairs were used to examine further if such N-transfer was also influenced by N_2-fixation. Two-way or net transfer was the sum or the difference, respectively, between N transferred to N_2-fixing plants and to non-N_2-fixing ones. To our knowledge, this is the first mycorrhiza-mediated two-way and net N-transfer study adopting such approaches, using ^{15}N labeling and/or $\delta^{15}N$ natural abundance analysis.

Through the CMNs between *Casuarina* and *Eucalyptus* or soybean and *Sorghum*, an artificial N concentration gradient between N-donor and N-receiver was established by depriving the N-receiver of the external N-supplement and enabling detection of the N isotope received. The results of the ^{15}N labeling experiments in Chapter 3 showed that N-transfer between *C. cunninghamiana* and *E. maculata* occurred bidirectionally in younger and older seedlings with all four different forms of ^{15}N

label. Surprisingly, there was a net gain in N only by the N_2-fixing *C. cunninghamiana*, but not by the non-N_2-fixing *E. maculata*, in seven out of eight combinations, the exception being the 6-months-old sole mycorrhizal pairs with $(^{15}NH_4)_2SO_4$.

The results of the ^{15}N natural abundance experiments in Chapter 4 confirmed that mycorrhiza-mediated N-transfer was bidirectional, and that a net gain in N was achieved only by the N_2-fixing *C. cunninghamiana* and soybean, but not by the non-N_2-fixeing *E. maculata* and *Sorghum*. Strikingly, the percentage and amount of N-transfer were also significant from *Eucalyptus* to nodulated *Casuarina*, or from *Sorghum* to soybean (with up to 50% or 40% biological N_2-fixation for *Casuarina* and soybean, respectively) rather than the reverse, contradicting the general hypothesis that N flows from the N_2-fixing plants to the non-N_2-fixing ones (Bethlenfavay *et al.*, 1991; Frey and Schuepp, 1993). However, the %NDFT was on the same scale regardless of the direction of N-transfer and was on average 30% for the four species, similar to the ~50% found in *Cichorium intybus* from *Pisum sativum* or *Trifolium pratense* via AM connections (Martensson *et al.*, 1998). Significantly higher bidirectional and net N-transfer was also found between the sole mycorrhizal and the dual nodulated/mycorrhizal pairs for both ^{15}N labeling and $\delta^{15}N$ natural abundance experiments.

The similar amounts of N transferred to *Eucalyptus* and to *Casuarina* (Table 3.7) or to *Sorghum* and to soybean (Table 4.15) in the sole mycorrhizal pairs suggests that two-way N-transfer can occur naturally between any mycorrhizal plants, regardless of whether they are N_2-fixing plants and/or non-N_2-fixing ones, and that N_2-fixation is not necessarily a prerequisite for N-transfer. It also suggests that the N resource could equally be reallocated between plants through mycorrhizal mediation, as generally no significant tissue N concentration difference was found in either the (non-nodulated) non-mycorrhizal or the (non-nodulated) mycorrhizal seedlings of the four species (data not shown). Such N circulation and equalisation would enable net translocation of N from areas of high N availability to areas of high N demand between two adjacent plants through the below-ground common mycorrhizal linkage. Should this phenomenon prove to be widespread, it may reduce resource

competition among the same or different species, and thus enhance coexistence and stability of plant communities (Miller and Allen, 1992; Read, 1992).

Moreover, the significantly greater intensity of bidirectional N-transfer in the nodulated mycorrhizal pairs indicates that more substantial amounts of N can be shuttled between plants. More important, a greater amount of net N transferred to the N_2-fixing plants from the non-N_2-fixing plants suggested that it is this N that can give further support to the N_2-fixing plants which have a generally greater physiological and ecological N requirement in low-external-N-input conditions (Subbarao and Rodrigue-Barruco, 1995; Williams and Woinarski, 1997). N_2-fixing plants may depend more on ECM or AM colonisation for their higher energy and P requirements for optimal N_2-fixing bacterial activity, than their adjacent non-N_2-fixing partners which lack such activity (Hayman, 1986; Smith and Read, 1997). Phosphorus demand is likely to be different for *Casuarina* and soybean (Reddell *et al.*, 1997b). Some *Casuarina* species can form cluster roots for P mobilization in extremely N-deficient soils (Subbarao and Rodrigue-Barruco, 1995; Neumann and Martinoia, 2002). However, cluster roots were not observed in *C. cunninghamiana* because growth conditions were optimal in the glasshouse experiments conducted in this study. ECM association may even be a facilitator for *Frankia* nodulation in *Casuarina*, as it has been shown that AM colonisation may be a prerequisite for *Rhizobium* nodulation in various legumes (Hayman, 1986). Besides root N access from soils, the experiments above suggest that *Casuarina* and soybean have two strategies, N_2-fixation and mycorrhization, to satisfy their high N-demand, while mycorrhization alone meets the needs for the relatively low N-demand by *Eucalyptus* and *Sorghum*. These two strategies clearly lead to a more profound net N gain by the N_2-fixing *Casuarina* and soybean than by the non-N_2-fixing *Eucalyptus* and *Sorghum*, as is clearly shown in this study. In addition, the following factors could have affected N-transfer between plants: swap of animal-origin N from soil arthropods in exchange for photosynthetic carbon in mycorrhizal plants (Klironomos and Hart, 2001) or N transferred from soil nematodes to plants through a mycorrhizal mycelial network (Perez-Moreno and Read, 2001). However, neither soil arthropods nor nematodes were investigated in this study.

The up to 13% N-transfer in ^{15}N labeling experiments and 24% N-transfer in the ^{15}N natural abundance experiments to *Eucalyptus* and the up to 21% N-transfer to *Casuarina* in both the ^{15}N labeling and the ^{15}N natural abundance experiments represent substantial N gains by both species (Tables 3.7 and 4.14), as were the 10% and 24% N-transfers in the pure systems (Table 4.12). There was no such previous report of N-transfer between the same species. The values are very similar to the ~20% one-way N-transfers measured in the Northern Hemisphere trees *Alnus* and *Pinus* (Arnebrant *et al.*, 1993; Ekblad and Huss-Danell, 1995). By comparison, the N-transfer to nodulated ectomycorrhizal casuarinas of up to 41% was relatively much higher (Table 3.7), as was the 32% N-transfer in nodulated mycorrhizal *Casuarina/Casuarina* pairs (Table 4.12). On the other hand, Table 4.14 also showed up to 23% or 29% N-transfer to AM *Sorghum* or soybean in the ^{15}N natural abundance experiment. These N transfers are higher than maximally 20% one-way AM-mediated N-transfers in many previous observations (Haystead *et al.*, 1988; Eissenstat, 1990; Frey and Schuepp, 1992, 1993; Ikram *et al.*, 1994; Martin *et al.*, 1995; Johansen and Jensen, 1996).

Nitrogen transfer of this magnitude between both ECM and AM plants could be biologically significant and affect growth performance of 'the receivers' under poor N conditions in the nutrient-impoverished Australian soils if N-transfer was coupled with a comparable C transfer. Bidirectional C transfer between *Betula papyrifera* and *Pseudotsuga menziesii* has been measured (Simard *et al.*, 1997a, c). It was observed that N transfers correlated with both greater biomass production and N content in the nodulated and the older seedlings in the ^{15}N labeling experiments (Figure 3.11) and in the ^{15}N natural abundance experiments (Figures 4.9, 4.14A - B, 4.22). In the latter, longer root interspecific hyphal connections were associated with increasing vigour of seedlings, indicating that transfer varied with seedling performance and environmental conditions. These data also showed that biomass increased with N content, and that mycorrhization had a remarkable effect on biomass production in both species.

However, in general, mycorrhization had little impact on N accumulation in *Eucalyptus* or *Sorghum*, while it had a major effect in *Casuarina* or soybean, despite the fact that *Eucalyptus* had nearly double the rate of infection compared with

Casuarina, and *Sorghum* had a relatively higher root colonisation. The nodulated mycorrhizal *Casuarina* or soybean and their companion mycorrhizal *Eucalyptus* or *Sorghum*, had the highest biomass yield and N content, respectively. This was also reflected in both biomass production and total N content in all the N-receivers that equaled these in the N-donors. Even better performance was achieved in the short term in the 6-months-old nodulated mycorrhizal 'N-receiver' casuarinas (Figure 3.11). These results support previous reports that the main effect of mycorrhization on plant N status was in the N_2-fixing plants (Hayman, 1986). They also provide a possible explanation that different N_2-fixing plants maintained negative associations with each other, while positive associations have been observed between an N_2-fixing plant and a non-N_2-fixing one (Turkington *et al.*, 1977).

5.5. Conclusions

This is the first report of the low-N-demanding non-N_2-fixing *Eucalyptus* and *Sorghum* supplying and thus nurturing the high-N-demanding N_2-fixing *Casuarina* and soybean. A possible suggestion is that mycorrhiza-associated non-N_2-fixing plants, more specifically their symbiotic fungus-root associations, can become 'N-donors' because of a lower N requirement, and that the adjacent partner N_2-fixing plants can be 'N-receivers' because of a higher N demand. It seems mycorrhization, together with N availability and requirements, and not the N_2-fixing nature of the plants, play a vital and decisive role in the re-distribution and flux direction of N between plants. Considering that N (either excess or deficiency) is more crucial to most terrestrial ecosystems than any other mineral element (Vitousek *et al.*, 1997; Moffat, 1998), and that the potential benefit of N-transfer management in soil-plant systems is great [CSIRO Publishing (Special issue), 1998], the significance of two-way N-transfer warrants investigation in more species and under real field conditions. The latter may not be practicable because of unavoidable NO_3^- diffusion and water movement.

Previous reports have shown that mycorrhizae can alter the quantity and quality of C allocated below-ground (Rygiewicz and Anderson, 1994; Simard *et al.*, 1997b) and the results reported in this thesis concerning N metabolism are consistent with this scenario. The extra C and N supplied via C and N transfer could provide mycorrhizal

plants with a competitive advantage over the non-mycorrhizal plants. In addition, greater N cycling between and within plants would reduce N loss through volatilization (NH_3 or N oxides) or through NO_3^- leaching to surface or groundwater (Janzen and Gilbertson, 1994; Vitousek *et al.*, 1997). Mycorrhization could thus help maintain N bioavailability and soil fertility in the long term. Consequently, these plant-to-plant N and C movements via the CMN could have important implications for terrestrial plant biodiversity, ecosystem stability and productivity (Miller and Allen, 1992; Read, 1992; Rygiewicz and Anderson, 1994; Tilman *et al.*, 1996).

This study has shown conclusively that N can be shuttled between mycorrhizal plants as a shared resource, and that the intensity of the N-transfer is affected by N_2-fixation. Depending on an established N source-sink relationship, it can be argued that any plant is capable of delivering N to, or receiving N from, an adjacent or neighbouring plant, if they are interconnected by a CMN. N_2-fixation capability may not be a prerequisite for N movement between plants, but N_2-fixing capacity may affect the magnitude of N-transfer. Therefore, the conventional definition of the N_2-fixing plant as an 'N-donor' and the non-N_2-fixing plant as an 'N-receiver' should be applied with great caution. Mycorrhiza-mediated N-transfer and N-cycling appear to be more complex and dynamic processes than many researchers and textbooks suggest. Two-way N-transfer provides important data to inform theoretical speculation that, in turn, will hopefully identify new questions for observational and experimental studies. In Figure 5.1 two 'mycocentric' N-transfer mechanisms are proposed to account for these differences. It seems that any plant that gives more N than it receives is an 'N-donor'; while the opposite is true for an 'N-receiver'. If these mechanisms operate as this study has demonstrated and prove to be widespread, ideas about mycorrhiza-mediated N exchange and cycling in both agricultural and natural ecosystems may have to be re-evaluated. Combined with concurrent C and P transfer through the CMN, concepts about nutrient cycling and energy exchange in plant communities may also have to be reformulated. The results from this study certainly have important implications for the N economy of N_2-fixing-based agricultural and natural ecosystems. In such ecosystems, the magnitude of the mycorrhiza-mediated N-transfer and N movement seems to be determined by the dynamic four-way interactions between plant roots, mycorrhizal fungi, N_2-fixing bacteria, and N resource availability and plant requirements.

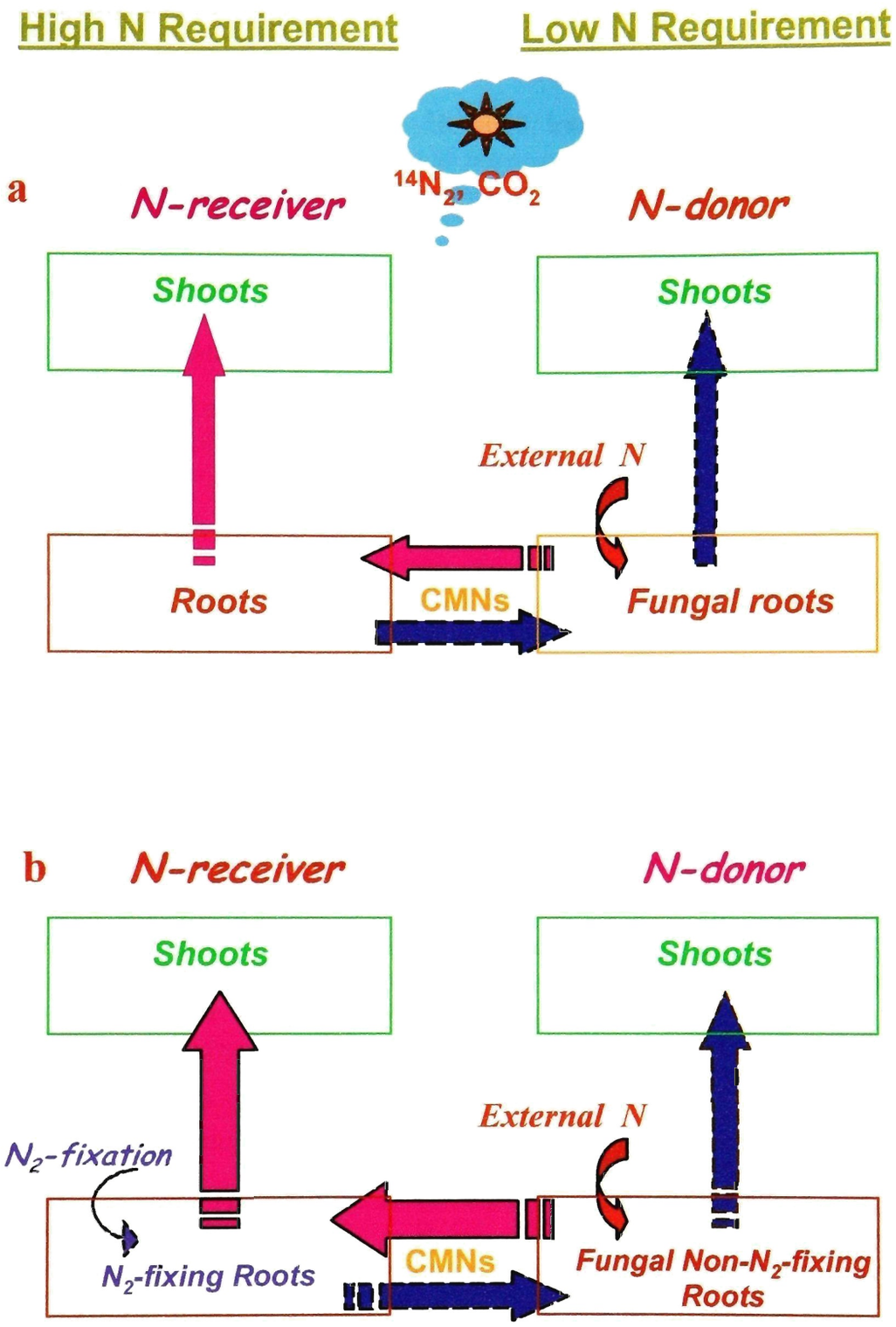

Figure 5.1. Conceptional diagrams of underground N shuttling between N-donor and N-receiver plants through common mycorrhizal networks (CMNs) (the arrow width indicates the relative magnitude of N-flux when the N-donor side has a higher concentration of N). a. Mycorrhiza-mediated N-transfer between non-N_2-fixing plants. b. Mycorrhiza-mediated N-transfer between a non-N_2-fixing and an N_2-fixing plant.

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Appendix 1. Dry weight, N content, $\delta^{15}N$ (‰) and atom%^{15}N in ^{15}N labeling experiments

Labeling with $(^{15}NH_4)_2SO_4$)

Treatments	DW (g/plant)			N (mg/plant)			$\delta^{15}N$ ‰	atom% ^{15}N	
	Shoot	Root	Nodule	Shoot	Root	Nodule	Shoot	Shoot	Root
*Cas1 control	18.46	6.83		165.14	53.75		1.62	0.5492	1.742
*Cas2 mycorrhiza	19.92	8.26		262.10	75.04		1.42	0.5458	1.579
*Cas3 mycorrhiza	20.12	9.12		271.75	88.66		0.88	0.5456	1.393
*Cas4 frankia	23.62	9.48	2.55	474.90	121.49	54.27	-0.02	0.5450	1.045
*Cas5 frankia + mycorrhiza	24.11	10.75	3.08	565.72	136.49	69.89	-0.51	0.5268	0.954
Cas: Casuarina									
Euc1 control	13.02	4.40		113.58	34.44		3.16	0.3675	0.381
Euc2 control	15.61	6.14		164.17	46.75		2.84	0.3677	0.383
Euc3 mycorrhiza	15.97	6.33		173.33	46.89		2.11	0.3683	0.397
Euc4 mycorrhiza	18.49	7.45		195.88	59.55		2.10	0.3698	0.401
Euc5 mycorrhiza	19.17	8.20		227.52	65.95		1.97	0.3701	0.417
Euc: Eucalyptus									
*Euc1 control	17.01	7.29		156.73	53.58		2.88	0.9450	1.996
*Euc2 mycorrhiza	19.21	7.81		196.19	60.75		2.22	0.7651	1.947
*Euc3 mycorrhiza	20.13	8.26		205.06	61.20		1.92	0.5872	1.707
*Euc4 mycorrhiza	21.17	8.43		231.76	66.80		1.81	0.5217	1.429
*Euc5 mycorrhiza	23.77	9.62		263.09	77.28		1.76	0.4735	1.215
Cas1 control	14.24	5.85		119.64	50.20		1.52	0.3666	0.376
Cas2 control	17.83	6.36		175.09	56.00		1.36	0.3668	0.373
Cas3 mycorrhiza	18.98	7.66		201.70	74.49		0.96	0.3675	0.378
Cas4 frankia	22.00	8.66	2.69	416.20	103.65	54.58	-0.10	0.3693	0.398
Cas5 frankia + mycorrhiza	23.53	9.43	2.97	544.08	114.28	72.73	-0.71	0.3710	0.451

Labeling with $K^{15}NO_3$

*Cas1 control	16.03	7.81		153.02	69.37		1.57	0.7351	2.7455
*Cas2 mycorrhiza	18.93	8.40		211.23	78.22		1.32	0.6207	2.0668
*Cas3 mycorrhiza	19.70	8.42		235.85	79.68		1.11	0.6021	1.8520
*Cas4 frankia	24.61	9.35	2.81	475.73	104.07	51.13	-0.74	0.4812	1.6114
*Cas5 frankia + mycorrhiza	25.65	10.83	3.50	549.05	123.70	64.06	-1.01	0.4954	1.3218
Euc1 control	14.58	4.56		103.76	32.76		2.22	0.3725	0.3899
Euc2 control	16.38	5.27		159.49	35.79		1.88	0.3676	0.4034
Euc3 mycorrhiza	17.31	5.56		175.23	41.66		1.72	0.3686	0.4166
Euc4 mycorrhiza	18.65	6.37		191.78	48.24		1.45	0.3696	0.4715
Euc5 mycorrhiza	19.91	7.72		216.28	51.16		1.33	0.3712	0.4773
*Euc1 control	15.82	5.46		161.53	43.90		2.35	0.9920	2.9128
*Euc2 mycorrhiza	17.76	6.30		180.29	53.83		1.84	0.8309	2.6133
*Euc3 mycorrhiza	19.10	6.79		206.20	57.62		1.73	0.7194	2.3066
*Euc4 mycorrhiza	20.28	7.10		229.33	65.06		1.51	0.6681	2.0046
*Euc5 mycorrhiza	22.00	8.45		266.84	74.50		1.46	0.5902	1.8341
Cas1 control	10.96	4.86		107.44	43.03		1.60	0.3670	0.3984
Cas2 control	15.86	6.48		143.68	60.76		1.44	0.3680	0.4050
Cas3 mycorrhiza	16.71	6.68		181.68	65.29		1.25	0.3698	0.4126
Cas4 frankia	21.68	9.30	2.40	412.43	111.64	58.00	-0.62	0.3749	0.4729
Cas5 frankia + mycorrhiza	22.49	9.77	2.97	572.67	130.56	79.22	-1.01	0.3785	0.4999

Appendix 1. (continued)

Labeling with $^{15}NH_4NO_3$

Treatments	DW (g/plant)			N (mg/plant)			$\delta^{15}N$ ‰	atom% ^{15}N	
	Shoot	Root	Nodule	Shoot	Root	Nodule	Shoot	Shoot	Root
*Cas1 control	36.53	11.60		263.54	101.57		2.52	0.5141	5.4834
*Cas2 mycorrhize	48.38	17.92		581.89	193.43		1.57	0.4538	3.2385
*Cas3 mycorrhiza	50.05	18.63		667.79	201.75		0.85	0.4362	3.1052
*Cas4 frankia	60.10	25.27	3.76	977.03	296.61	62.74	0.35	0.4218	2.6622
*Cas5 frankia + mycorrhize	66.22	28.40	4.99	1248.76	351.59	86.89	-0.16	0.4066	1.8064
Cas: Casuarina									
Euc1 control	22.43	7.02		115.35	41.30		4.61	0.3704	3.3159
Euc2 control	27.86	11.63		154.09	69.20		4.02	0.3717	0.5214
Euc3 mycorrhiza	28.33	12.01		178.97	75.34		3.87	0.3731	0.7270
Euc4 mycorrhiza	31.60	14.81		225.80	101.60		3.81	0.3747	0.9231
Euc5 mycorrhiza	32.49	15.07		272.20	121.00		3.64	0.3862	0.9309
Euc: Eucalyptus									
*Euc1 control	24.43	8.49		145.08	56.57		5.06	0.9106	2.3366
*Euc2 mycorrhiza	29.52	12.30		181.83	76.40		4.18	0.5701	1.9414
*Euc3 mycorrhiza	30.80	12.75		199.11	82.24		3.94	0.5016	1.6038
*Euc4 mycorrhiza	32.85	14.60		301.81	110.52		3.69	0.4786	1.4270
*Euc5 mycorrhiza	33.76	15.62		332.62	124.56		3.42	0.4160	1.2060
Cas1 control	33.90	10.50		273.98	88.38		2.37	0.3678	0.3805
Cas2 control	43.79	16.75		443.91	170.45		1.67	0.3683	0.4484
Cas3 mycorrhiza	44.82	17.22		553.87	185.90		0.82	0.3699	0.4686
Cas4 frankia	54.31	23.97	4.33	924.27	257.77	77.27	-0.13	0.3719	0.7222
Cas5 frankia + mycorrhiza	56.48	25.23	5.85	1101.84	299.12	101.34	-0.69	0.3770	0.8358
Labeling with $NH_4{}^{15}NO_3$									
*Cas1 control	34.48	11.09		248.81	111.01		2.52	0.6115	8.6912
*Cas2 mycorrhiza	48.25	17.74		602.54	180.60		1.61	0.5329	6.4293
*Cas3 mycorrhiza	49.53	18.57		671.40	194.87		0.74	0.4949	5.8746
*Cas4 frankia	60.48	27.13	3.62	966.99	311.88	77.53	0.12	0.4868	5.4852
*Cas5 frankia + mycorrhiza	64.09	28.87	4.67	1195.25	323.79	92.44	-0.30	0.4770	3.6371
Euc1 control	21.82	6.96		127.97	40.13		4.89	0.3730	0.5951
Euc2 control	27.77	11.80		188.14	77.64		4.22	0.3747	0.6584
Euc3 mycorrhiza	28.37	12.42		202.74	86.13		3.91	0.3788	0.6735
Euc4 mycorrhiza	31.53	15.33		223.33	105.86		3.81	0.3898	0.8904
Euc5 mycorrhiza	32.97	15.90		270.35	115.04		3.38	0.4047	1.0951
*Euc1 control	22.73	8.12		157.30	58.96		5.07	0.9460	3.1057
*Euc2 mycorrhiza	29.60	13.74		216.49	85.11		4.52	0.7435	2.7686
*Euc3 mycorrhiza	30.27	14.36		228.25	97.08		4.46	0.6541	2.5380
*Euc4 mycorrhiza	32.52	15.50		332.11	119.62		3.86	0.5082	1.8171
*Euc5 mycorrhiza	34.72	16.73		356.56	131.61		3.46	0.4662	1.6878
Cas1 control	33.83	10.73		241.93	93.95		2.57	0.3674	0.3982
Cas2 control	44.90	17.60		463.88	172.07		1.60	0.3712	0.4089
Cas3 mycorrhiza	45.80	18.79		542.04	193.14		0.69	0.3764	0.4445
Cas4 frankia	56.83	23.95	4.18	877.20	268.02	79.50	-0.12	0.3857	0.5583
Cas5 frankia + mycorrhiza	58.53	28.48	5.58	1039.14	291.39	100.76	-0.79	0.3965	0.7762

Appendix 2. Dry weight, N content and $\delta^{15}N$ in ^{15}N natural abundance experiments

a. Nitrogen transfer between *Eucalyptus/Eucalyptus* (N-plus experiment)

	DW (g/plant)		N (mg/plant)		^{15}N (‰)	
Treatment	Shoot	Root	Shoot	Root	Shoot	Root
EucalyptusA $_{control}$	36.24	13.63	229.81	63.83	2.57	1.81
EucalyptusA $_{mycorrhiza}$	46.49	22.78	650.60	147.84	2.33	1.62
EucalyptusA $_{mycorrhiza}$	47.82	25.06	711.42	168.24	2.36	1.62
EucalyptusB $_{control}$	36.71	13.98	212.20	64.96	2.57	1.80
EucalyptusB $_{-mycorrhiza}$	45.45	22.17	622.97	134.00	2.34	1.60
EucalyptusB $_{mycorrhiza}$	47.05	24.15	706.25	170.15	2.35	1.60

Appendix 2. (continued)

b. Nitrogen transfer between *Casuarina/Casuarina* (N-plus experiment)

	DW (g/plant)			N (mg/plant)			^{15}N (‰)		
Casuarina A/B	Shoot	Root	Nodule	Shoot	Root	Nodule	Shoot	Root	Nodule
CasA1 $_{control}$	61.04	33.12		553.21	260.42		0.84	1.16	
CasA2 $_{frankia}$	99.25	50.25	8.57	1923.10	553.21	125.79	-2.16	-0.26	2.64
CasA3 $_{mycorrhizae}$	84.36	41.96		995.21	431.21		-0.87	0.48	
CasA4 $_{frankia}$	117.87	59.10	11.12	2479.64	676.54	186.47	-2.43	-0.31	2.27
CasA5 $_{frankia + mycorrhiza}$	119.76	60.10	11.96	2694.76	694.03	208.70	-2.59	-0.51	2.30
Cas: Casuarina									
CasB1 $_{control}$	59.21	29.18		542.31	250.30		0.80	1.04	
CasB2 $_{-mycorrhiza}$	59.77	29.51		567.69	255.77		0.82	1.14	
CasB3 $_{-mycorrhiza}$	82.12	38.63		948.83	367.40		-0.81	0.47	
CasB4 $_{mycorrhiza}$	85.49	40.39		993.83	387.99		-1.87	-0.18	
CasB5 $_{frankia}$	118.08	59.37	10.57	2446.88	667.04	184.64	-2.41	-0.25	2.23
Casuarina X/Y									
CasX1 $_{control}$	60.54	33.08		554.41	283.97		0.85	1.17	
CasX2 $_{-mycorrhiza}$	60.43	33.56		573.93	290.82		0.82	1.20	
CasX3 $_{-mycorrhiza}$	83.62	41.08		944.28	427.42		-0.82	0.49	
CasX4 $_{mycorrhiza}$	84.76	41.20		996.56	438.41		-1.91	-0.27	
CasX5 $_{frankia}$	119.89	60.96	11.72	2538.07	731.62	205.23	-2.46	-0.52	2.27
CasY1 $_{control}$	60.34	30.51		546.83	240.66		0.81	1.08	
CasY2 $_{frankia}$	99.74	49.95	8.49	1908.44	516.24	125.79	-2.18	-0.27	2.58
CasY3 $_{mycorrhiza}$	83.34	40.48		975.68	397.38		-0.84	0.47	
CasY4 $_{frankia}$	115.09	58.56	10.83	2362.51	662.50	183.61	-2.48	-0.17	2.28
CasY5 $_{frankia + mycorrhiza}$	120.29	60.64	12.55	2748.27	710.61	222.35	-2.57	-0.25	2.24

Appendix 2. (continued)

c. Nitrogen transfer between *Casuarina* and *Eucalyptus* (N-plus experiment)

	DW (g/plant)			N (mg/plant)			^{15}N (‰)		
Treatment	Shoot	Root	Nodule	Shoot	Root	Nodule	Shoot	Root	Nodule
CasA1 $_{control}$	59.59	29.24		422.48	249.78		0.16	1.43	
CasA2 $_{mycorrhiza}$	82.65	39.31		902.45	397.38		-1.08	0.75	
CasA3 $_{frankia}$	99.72	49.33	9.04	1613.11	554.50	122.19	-1.81	0.46	2.23
CasA4 $_{frankia + mycorrhiza}$	117.05	58.63	12.62	2328.91	691.70	205.82	-2.14	0.39	1.99
Cas: *Casuarina*									
EucA1 $_{control}$	35.43	13.69		233.08	63.43		2.64	1.95	
EucA2 $_{-mycorrhizae}$	45.35	22.11		623.52	156.08		2.39	1.74	
EucA3 $_{-mycorrhiza}$	36.09	14.30		245.23	75.27		2.33	1.65	
EucA4 $_{-mycorrhiza}$	46.11	22.92		654.47	162.15		2.23	1.31	
Euc: *Eucalyptus*									
EucB1 $_{control}$	35.50	13.72		233.55	63.56		2.69	1.96	
EucB2 $_{-mycorrhiza}$	36.17	14.33		245.72	75.42		2.38	1.66	
EucB3 $_{mycorrhiza}$	46.56	23.26		677.77	164.73		2.25	1.70	
EucB4 $_{mycorrhiza}$	48.66	24.98		758.14	196.51		2.16	1.27	
CasB1 $_{control}$	59.47	29.18		421.63	249.29		0.16	1.44	
CasB2 $_{frankia}$	99.52	49.23	9.04	1609.89	553.39	122.19	-1.81	0.47	2.27
CasB3 $_{-mycorrhiza}$	78.13	38.65		731.13	392.37		-0.45	1.01	
CasB4 $_{frankia}$	114.54	58.37	11.36	2252.74	686.75	188.02	-2.19	0.33	1.96

d. Nitrogen transfer between *Casuarina* and *Eucalyptus* (N-minus experiment)

	DW (g/plant)			N (mg/plant)			^{15}N (‰)		
Treatment	Shoot	Root	Nodule	Shoot	Root	Nodule	Shoot	Root	Nodule
CasA1 $_{control}$	7.46	3.16		98.85	29.81		1.01	0.51	
CasA2 $_{mycorrhiza}$	7.69	3.33		136.48	35.78		0.46	0.26	
CasA3 $_{frankia}$	9.66	4.10	1.18	200.36	48.50	25.16	0.28	-0.04	1.43
CasA4 $_{frankia + mycorrhiza}$	11.66	5.01	1.44	264.46	64.43	32.36	0.17	-0.40	1.69
Cas: *Casuarina*									
EucA1 $_{control}$	3.81	1.73		19.14	11.70		1.13	0.76	
EucA2 $_{-mycorrhiza}$	4.13	1.78		22.91	12.75		0.95	0.69	
EucA3 $_{-mycorrhiza}$	3.93	1.77		20.04	12.33		1.17	0.77	
EucA4 $_{-mycorrhiza}$	5.24	2.54		36.13	21.92		0.88	0.60	
Euc: *Eucalyptus*									
EucB1 $_{control}$	3.83	1.74		19.24	11.76		1.19	0.86	
EucB2 $_{-mycorrhiza}$	3.95	1.78		20.14	12.39		1.21	0.77	
EucB3 $_{mycorrhiza}$	4.24	1.81		23.87	13.50		0.97	0.60	
EucB4 $_{mycorrhiza}$	5.51	2.70		39.14	23.55		0.85	0.58	
CasB1 $_{control}$	7.42	3.15		98.36	29.67		0.95	0.50	
CasB2 $_{frankia}$	9.62	4.08	1.18	199.36	48.26	25.16	0.30	-0.04	1.43
CasB3 $_{-mycorrhiza}$	7.55	3.20		125.23	32.72		0.42	0.28	
CasB4 $_{frankia}$	11.28	4.82	1.39	254.95	60.06	30.97	0.19	-0.38	1.51

Appendix 2. (continued)

e. Nitrogen transfer between soybean and Sorghum (N-minus experiment)

	DW (g/plant)			N (mg/plant)			^{15}N (‰)		
Treatment	Shoot	Root	Nodule	Shoot	Root	Nodule	Shoot	Root	Nodule
soyA1 $_{control}$	1.09	0.24		25.94	4.07		7.59	5.21	
soyA2 $_{mycorrhiza}$	1.66	0.52		50.33	10.48		6.39	4.42	
soyA3 $_{rhizobia}$	2.23	0.80	0.16	76.29	17.08	8.07	6.01	4.03	5.21
soyA4 $_{rhizobia + mycorrhiza}$	2.32	0.87	0.17	88.96	19.54	9.16	5.35	3.62	5.11
soy: soybean									
SorA1 $_{control}$	1.87	0.85		25.19	6.47		8.78	4.89	
SroA2 $_{-mycorrhiza}$	2.73	1.50		45.37	12.82		7.55	4.22	
SorA3 $_{-mycorrhiza}$	1.96	0.86		28.53	6.89		7.71	4.37	
SorA4 $_{-mycorrhiza}$	3.84	1.96		70.81	20.66		7.05	3.64	
Sor: Sorghum									
SorB1 $_{control}$	1.87	0.85		25.24	6.50		8.96	4.99	
SorB2 $_{-mycorrhiza}$	1.96	0.86		28.59	6.92		7.86	4.45	
SorB3 $_{mycorrhiza}$	2.91	1.56		50.46	14.67		7.64	4.19	
SorB4 $_{mycorrhiza}$	3.96	2.02		73.54	22.10		7.13	3.40	
soyB1 $_{control}$	1.09	0.24		25.89	4.05		7.55	5.18	
soyB2 $_{rhizobia}$	2.23	0.80	0.16	76.14	16.99	8.22	5.98	4.01	5.21
soyB3 $_{-mycorrhiza}$	1.11	0.26		26.77	4.98		6.47	4.66	
soyB4 $_{rhizobia}$	2.27	0.83	0.16	78.35	18.08	9.67	5.26	3.78	5.39

Appendix 3. Chlorophyll content in plants

N-transfer between Casuarina/Eucalyptus, and soybean/Sorghum (N-minus experiments)

Treatment	Chlorophyll (µg/gDW) Chl *a*	Chl *b*	Chl *a+b*	Treatment	Chlorophyll (µg/gDW) Chl *a*	Chl *b*	Chl *a+b*
CasA1 $_{control}$	57.35	28.03	85.38	*soyA1* $_{control}$	38.52	17.66	56.18
CasA2 $_{mycorrhiza}$	65.49	32.13	97.62	*soyA2* $_{mycorrhiza}$	57.16	27.28	84.44
CasA3 $_{frankia}$	94.26	46.22	140.48	*soyA3* $_{rhizobia}$	82.25	41.02	123.27
CasA4 $_{frankia + mycorrhiza}$	122.80	58.80	181.60	*soyA4* $_{rhizobia + mycorrhiza}$	91.53	44.20	135.73
				***soy**: soybean*			
EucA1 $_{control}$	101.89	45.08	146.97	*SorA1* $_{control}$	52.91	22.48	75.39
EucA2 $_{-mycorrhiza}$	108.38	53.21	161.59	*SroA2* $_{-mycorrhiza}$	79.86	35.87	115.74
EucA3 $_{-mycorrhiza}$	105.07	52.13	157.21	*SorA3* $_{-mycorrhiza}$	53.21	22.85	76.06
EucA4 $_{-mycorrhiza}$	164.36	80.42	244.78	*SorA4* $_{-mycorrhiza}$	112.30	52.55	164.85
				***Sor**: Sorghum*			
EucB1 $_{control}$	102.40	45.31	147.70	*SorB1* $_{control}$	53.17	22.59	75.39
EucB2 $_{-mycorrhiza}$	105.59	52.39	157.98	*SorB2* $_{-mycorrhiza}$	53.47	22.96	76.06
EucB3 $_{mycorrhiza}$	116.34	56.28	172.63	*SorB3* $_{mycorrhiza}$	83.26	36.77	119.44
EucB4 $_{mycorrhiza}$	171.37	84.96	256.32	*SorB4* $_{mycorrhiza}$	115.72	55.77	170.64
CasB1 $_{control}$	57.06	27.89	84.95	*soyB1* $_{control}$	38.32	17.57	56.18
CasB2 $_{frankia}$	93.79	45.98	139.77	*soyB2* $_{rhizobia}$	81.83	40.81	123.26
CasB3 $_{-mycorrhiza}$	58.09	28.45	86.54	*soyB3* $_{-mycorrhiza}$	39.14	18.50	57.93
CasB4 $_{frankia}$	114.02	56.68	170.70	*soyB4* $_{rhizobia}$	82.65	42.84	126.12

***Cas**: Casuarina*

***Euc**: Eucalyptus*

Appendix 4. Amino acid composition in stem and root xylem sap

	Animo acid (μmolN/ml)															
	Glu+Gln		Asp+Asn		Ser+Ala		GABA		Proline		Cirtrulline		Others		Total	
Treatments	Stem	Root	Stem	Root	Stem	Root	Stem	Root	Stem	Root	Stem	Root	Stem	Root	Stem	Root
CasA1 control	277	76	243	14	187	107	120	50	644	523	501	38	205	87	2177	895
CasA2 mycorrhiza	250	116	232	24	272	157	91	108	983	772	631	487	323	138	2782	1802
CasA3 frankia	256	305	201	181	206	238	100	79	1768	1591	558	343	246	251	3335	2988
CasA4 frankia + mycorrhiza	252	163	364	34	240	202	102	85	1467	1329	805	943	166	246	3396	3002
EucA1 control	26	8	13	0	32	20	14	9	0	12	4	0	29	12	136	53
EucA2 -mycorrhiza	59	14	0	1	50	18	20	8	10	15	5	0	25	16	155	57
EucA3 -mycorrhiza	43	9	0	0	46	15	18	5	17	26	6	0	26	8	139	51
EucA4 -mycorrhiza	60	43	0	0	64	46	22	18	16	0	0	0	37	40	199	170
EucB1 control	26	8	13	0	32	21	14	9	0	12	4	0	29	12	136	53
EucB2 -mycorrhiza	44	9	0	0	46	15	18	5	17	26	6	0	27	8	140	51
EucB3 mycorrhiza	45	11	1	0	48	14	20	5	0	12	5	0	24	27	157	61
EucB4 mycorrhiza	64	39	31	13	58	54	21	19	0	0	4	0	50	44	228	179
CasB1 control	276	75	242	14	186	106	119	50	641	520	498	38	204	87	2166	890
CasB2 frankia	255	303	200	180	205	237	99	79	1759	1583	555	341	245	250	3318	2973
CasB3 -mycorrhiza	215	129	122	26	190	162	107	86	1573	998	267	276	113	99	2587	1776
CasB4 frankia	290	177	274	37	228	207	113	82	1487	1421	571	892	378	158	3340	2973
soyA1 control	791	365	276	154	153	152	33	37					445	329	1698	1038
soyA2 mycorrhiza	1579	510	572	463	420	490	181	90					1194	511	3946	2063
soyA3 rhizobia	1977	1726	867	443	483	319	73	28					1998	657	5398	3173
soyA4 rhizobia + mycorrhiza	1663	1208	2491	832	556	409	128	90					1336	846	6174	3385
soy: soybean																
SorA1 control	60	24	267	336	94	53	133	107					281	127	835	646
SroA2 -mycorrhiza	79	96	450	461	119	104	368	147					317	269	1333	1077
SorA3 -mycorrhiza	43	76	236	235	69	93	251	148					313	207	912	759
SorA4 -mycorrhiza	175	56	628	643	121	119	229	233					651	311	1804	1361
Sor: Sorghum																
SorB1 control	61	24	268	337	94	53	134	108					280	126	839	650
SorB2 -mycorrhiza	44	77	237	236	69	94	252	149					312	206	917	763
SorB3 mycorrhiza	73	70	493	413	135	109	252	245					403	251	1360	1090
SorB4 mycorrhiza	156	182	645	666	126	118	275	123					672	357	1881	1449
soyB1 control	787	364	275	153	152	152	33	37					447	331	1689	1033
soyB2 rhizobia	1967	1718	863	441	481	317	73	28					2008	661	5371	3157
soyB3 -mycorrhiza	991	403	327	194	220	130	15	22					511	370	2060	1115
soyB4 rhizobia	2260	904	1586	1108	639	316	115	89					1407	831	5993	3240